UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA
DEPARTAMENTO DE QUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM QUÍMICA

Avaliação de diferentes estratégias de determinação de nutrientes e potenciais contaminantes em alimentos por técnicas baseadas em laser

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Tese apresentada como parte dos requisitos para obtenção do título de DOUTORA EM CIÊNCIAS, área de concentração: QUÍMICA ANALÍTICA.

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*Bolsista: FAPESP (Projetos 2014/11415-0 e 2016/17443-0)

São Carlos-SP
2018

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Exatas e de Tecnologia
Programa de Pós-Graduação em Química

Folha de Aprovação

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“The greatest happiness of life is the conviction that we are loved; loved for ourselves, or rather, loved in spite of ourselves.”

Victor Hugo

Dedico aos meus pais Wilson e Tereza
e ao meu irmão Fernando,
com amor sempre.

Agradecimentos

Agradeço a Deus pelas oportunidades e por me iluminar sempre e a minha família por todo amor, carinho, confiança e compreensão;

Ao Prof. Dr. Edenir pela orientação, amizade, conselhos, paciência e compreensão demonstradas durante meu mestrado e doutorado;

Ao Prof. Dr. Joaquim e Prof. Dra. Ana Rita pelo apoio, amizade, críticas e sugestões durante todos esses anos em que fiz parte do GAIA;

Ao Dr. Jhanis Gonzalez, Dr. Richard E. Russo, Dra. Vassilia Zorba e Dra. Dayana Oropeza pela ajuda, amizade e parceria durante o estágio no exterior no Lawrence Berkeley National Laboratory (Berkeley-CA, EUA);

Aos meus amigos William, Lorenzo, Francisco, Amanda, Rose, Laiane, Helena e Neto por todos os momentos de alegria e companheirismo durante o ano que estive na Califórnia, nunca esquecerei de vocês;

Aos meus amigos do GAIA e da vida: Juliana, Nayara, Jeyne, Daniel, Marco Aurélio, Raquel, Vinicius, Diego, Lucimar, Mateus, Ariane, Érica, Alex, Sidnei e Juan pelos anos de convivência, amizade, troca de experiências profissionais e carinho;

À Fapesp pelo apoio financeiro - FAPESP - 2014/11415-0 e FAPESP - 2016/17443-0 (estágio no exterior);

À CAPES, pois o presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001;

Aos membros do banca por aceitarem o convite para participar da avaliação desta tese de doutorado;

As editoras Elsevier, Springer Nature e Publicações SBQ (Sociedade Brasileira de Química) pela autorização para utilizar os artigos publicados e apresentados no corpo desta tese;

Obrigada!!!

This PhD thesis is based on the following publications and manuscript for publication, wich are presented in the original format:

"Calibration strategies for the direct determination of Ca, K, and Mg in commercial samples of powdered milk and solid dietary supplements using laser-induced breakdown spectroscopy (LIBS)"

Amanda dos Santos Augusto, Paulo Lopes Barsanelli, Fabíola Manhas Verbi Pereira, Edenir Rodrigues Pereira-Filho. *Food Research International* 94 (2017) 72–78.

"Nutrient and contaminant quantification in solid and liquid food samples using laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS): discussion of calibration strategies"

Amanda dos Santos Augusto, Marco Aurélio Sperança, Daniel Fernandes Andrade and Edenir Rodrigues Pereira-Filho. *Food Analytical Methods* 10 (2017) 1515-1522.

"Combination of multi-energy calibration (MEC) and laser-induced breakdown spectroscopy (LIBS) for dietary supplements analysis and determination of Ca, Mg and K"

Amanda dos Santos Augusto, Jeyne Pricylla Castro, Marco Aurelio Sperança, Edenir Rodrigues Pereira-Filho. *Journal of the Brazilian Chemical Society, DOI: http://dx.doi.org/10.21577/0103-5053.20180211.*

"Protocol proposition for the direct analysis of solid samples by Laser Ablation-Inductively Coupled Optical Emission Spectroscopy (LA-ICP OES) and Laser-Induced Breakdown Spectroscopy (LIBS): Powdered dietary supplements example"

Amanda dos Santos Augusto, Dayana Oropeza, Edenir R. Pereira-Filho, Jhanis Gonzalez, Richard E. Russo, Vassilia Zorba. *Submitted.*

LIST OF ACRONYNS

ANNs - Artificial neural networks

ANVISA - *Agência Nacional de Vigilância Sanitária*

BEC - Background Equivalent Concentration

CF-LIBS - Calibration Free-Laser-induced breakdown spectroscopy

DLLME - Dispersive Liquid–Liquid Micro Extraction

F AAS - Flame Atomic Absorption Spectrometry

FAO - Food and Agriculture Organization of the United Nations

HG-ICP-OES - Hydride generation inductively coupled plasma optical emission spectrometry

HR-CS GF AAS - Solid sampling high resolution continuum source graphite furnace atomic absorption spectrometry

fs-LA-TOF-ICP-MS - femtosecond Laser Ablation-Inductively Coupled Plasma Time-of-Flight Mass Spectrometry

IC–ICP-MS – Ion Chromatography – Inductively Coupled Plasma Mass Spectroscopy

ICP-MS - Inductively Coupled Plasma–Mass Spectrometry

ICP OES - Inductively Coupled Plasma Optical Emission Spectrometry

IDR - *Ingestão diária recomendada*

KNN - k-Nearest Neighbor

LA-ICP-MS - Laser Ablation Inductively Coupled Plasma Mass Spectrometry

LA-ICP OES - Laser Ablation Inductively Coupled Plasma Optical Emission Spectrometry

LIBS - Laser-Induced Breakdown Spectroscopy

LIF - Laser-Induced Fluorescence

LOD - Limit Of Detection

LOQ - Limit Of Quantification

MEC - Multi - Energy Calibration

MLR - Multiple linear regression

PCA - Principal Component Analysis

PCR - Principal Component Regression

PFA – Perfluoroalcoxi

PLS – DA - Partial Least Squares for Discriminant Analysis

PLS - Partial Least Squares

PVA - Polyvinyl Alcohol

RSD - Relative Standard deviation

SD - Standard deviation

SIMCA - Soft Independent Modelling of Class Analogy

UV-vis - Ultraviolet–visible spectroscopy

WHO - World Health Organization

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RESUMO

AVALIAÇÃO DE DIFERENTES ESTRATÉGIAS DE DETERMINAÇÃO DE NUTRIENTES E POTENCIAIS CONTAMINANTES EM ALIMENTOS POR TÉCNICAS BASEADAS EM LASER. Esta tese enfoca o desenvolvimento de estratégias para a determinação elementar de Ca, Cd, Cr, Cu, Fe, Mg, Pb e Zn em amostras de sucos de laranja e suplementos alimentares, por *Laser-Induced Breakdown Spectroscopy* (LIBS). Para a determinação dos analitos estudados em suplementos, por LIBS, foram avaliados diversos métodos de calibração como: a calibração externa onde usou-se a celulose e as próprias amostras como base para a produção de materiais de referência sólidos, a PLS (*Partial Least Squares*) como opção de calibração multivariada e a calibração denominada MEC (*Multi - Energy Calibration*). As amostras de suco e padrões aquosos foram imobilizados em um fino filme polimérico de PVA *(Polyvinyl Alcohol)* e analisados por LIBS e LA-ICP-MS. Nas análises por LIBS mesmo com o emprego da conversão da amostra do estado líquido para sólido e/ou do método DLLME (*Dispersive Liquid–Liquid Micro Extraction*), em diferentes suportes, não foi possível obter sinais de intensidade suficientemente intensos para os analitos estudados. Durante a realização deste projeto foi possível a realização de um estágio no exterior com duração de um ano no Lawrence Berkeley National Laboratory sob a supervisão do Dr. Richard Russo. Nesta parte do trabalho amostras de suplementos alimentares foram analisadas, porém utilizando um sistema tandem LIBS/LA-ICP OES (Laser Ablation Inductively Coupled Plasma Optical Emission) de forma a propor um protocolo de análise para análise direta de sólidos. A Quimiometria foi aplicada durante todo o projeto para otimizar as condições analíticas e identificar a melhores condições de trabalho. Ferramentas quimiométricas foram empregadas também para a geração de modelos de calibração visando à determinação expedita dos analitos.

ABSTRACT

EVALUATION OF DIFFERENT STRATEGIES FOR NUTRIENT AND POTENTIAL CONTAMINANTS DETERMINATION IN FOODS BY LASER-BASED TECHNIQUES. The present project focuses on the development of strategies for the elemental determination of Ca, Cd, Cr, Cu, Fe, Mg, Pb and Zn in samples of orange juice and dietary supplements by Laser-Induced Breakdown Spectroscopy (LIBS). For the determination of the analytes studied in supplements, by LIBS, several calibration methods were evaluated: the external calibration where cellulose was used and the samples themselves as the basis for the production of solid reference materials, PLS (Partial Least Squares) as a multivariate calibration option and the calibration called MEC (Multi - Energy Calibration). Juice samples and aqueous standards were immobilized on a thin polymer film of PVA (Polyvinyl Alcohol) and analyzed by LIBS and LA-ICP-MS. In the analyzes by LIBS even with the conversion of the sample from the liquid to the solid state and/or with the DLLME (Dispersive Liquid-Liquid Micro Extraction) method, in different supports, it was not possible to obtain signals of intensity sufficiently intense for the analyzed analytes. During the implementation of this project it was possible to hold a one-year internship at the Lawrence Berkeley National Laboratory under the supervision of Dr. Richard Russo. In this part of the work dietary supplements were analyzed, but using a LIBS / LA-ICP OES (Laser Ablation Inductively Coupled Plasma Optical Emission) in order to propose a protocol of direct analysis of solids. Chemometrics was applied throughout the project to optimize the analytical conditions and identify the best working conditions. Chemometric tools were also used to generate calibration models for the rapid determination of the analytes.

SUMMARY

Chapter 1 – Introduction

1. Introduction

A healthy and balanced diet contains twenty eight chemical elementals considered of extreme necessity for the proper function of the human organism. A diet rich in fruit juices, for example, is a good source of a wide range of physiologically and nutritionally important compounds, such as proteins, vitamins, pectins, flavonoids, phenolic acids, and macroelements such as Ca, K, Mg, P and S. Some micronutrients as Cr, Fe, Mg and Mo are commonly found in foods, most of which come from the cultivation soil where it can be also found toxic elements such as Cd and Pb from soil and production and due to the use of pesticides and fertilizers, for example [1]. According to ANVISA (*Agência Nacional de Vigilância Sanitária*) elements as Cd and Pb should not present concentrations higher than 0.05 mg kg^{-1} in fruit juices and nectars [2]. For nutrients such as Ca, Cu, Fe, Mg, P and Zn, the values indicated by ANVISA as a recommended daily intake for adults are: 800, 3, 14, 300, 800 and 15 mg, respectively. For Cr and Se these values are 200 and 70 μg, respectively [3].

In Brazil, besides of to nutritional importance, fruit juices, mainly orange juice also act as an important role in the citrus industry. Brazil ranks first worldwide and is responsible for 50% of the world production of FCOJ (Frozen Concentrate Orange Juice) and NFC (Not Frozen Concentrate) [4]. This large production indicates the importance of this type of product to the Brazilian economy, the composition of the GDP (Gross Domestic Product) and the supply, as well as the quality of the product marketed, should be prioritized. For these reasons, it is not surprising that juices are a subject of great concern and interest to many researchers, food analysts and nutritionists, who want to determine their nutritional quality and food safety.

Even with a healthy diet, sometimes the nutrients absorbed are insufficient. In this scenario, food supplements may be an alternative to reduce nutrient deficiency. The need for the use of supplements may be due to poor diet or to special cases such as pregnant women and children with a restrictive diet [5].

European Union (EU) countries [6] define food supplements as concentrated sources of vitamins, minerals and / or other substances (such as amino acids, fiber, various herbs and herbal extracts) marketed as pills, tablets, powder and other dosage forms.

This type of product is prepared synthetically in laboratories or using natural products such as plants and fish oils containing high concentrations of vitamins, minerals and other nutrients [5]. During the production process or even together with the raw material it is possible to occur the presence of toxic metals [7], therefore, like the food products, these compounds must have a strict control that guarantees its quality and safety in the possible presence of contaminants. In addition, the amount of nutrients should not exceed the required IDR (*Ingestão Diária Recomendada*) values.

Extrapolation of the limits of macroelements, trace elements and contaminants can moderately or severely affect the proper functioning of the organism leading to problems such as hypercalcemia (excess of Ca in the blood), gastrointestinal problems (excess Zn and Se), cancer (contamination by Cd and Pb), among others. According to the Codex Alimentarius the minimum level of each vitamin and / or minerals contained in food supplements per daily portion of consumption as suggested by the manufacturer should be 15% of the IDR. This value is determined by the FAO (Food and Agriculture Organization of the United Nations (FAO)/ WHO (World Health Organization) [8]. For EU the maximum permissible concentrations of elements such as Cd and Pb are 3 mg kg^{-1} [9] and for ANVISA the tolerable limit of Pb for special purpose foods is 0.2 mg kg^{-1} [10].

1.1 Nutrients and trace elements

A daily dose of macro and micronutrients is required for the proper functioning of the human organism. Some minerals generally found in foods are: Ca, K, Mg, Na (macro elements, i.e.> 0.01% or 100 mg kg^{-1}) and Cr, Cu, Fe, Mn, Se, Zn (trace elements, i.e. <0.01% or 100 mg kg^{-1}) [11]. About 99% of all Ca

present in the body resides within the hydroxyapatite skeleton. This element is involved in processes such as blood coagulation, nerve transmission and muscle contraction [12]. Its deficiency can cause chronic diarrhea, short bowel syndrome among others, since the excess of this in the organism can cause hypercalcemia that presents as neurological, renal and gastrointestinal problem symptoms [13]. Zn is more easily found in products of animal origin, being an efficient antioxidant and is present in processes such as protein synthesis. Zn deficiency impairs all synthesis processes and can cause gastrointestinal problems, fatigue and at high levels generate enlargement of the prostate and contribute to the risk of developing Alzheimer's disease [14].

Magnesium is a cofactor in over 300 enzymatic reactions and is present in all reactions involving the transfer of ATP (Adenosine Triphosphate) [15]. Mg deficiency in the human body can result into tumors and cardiac arrhythmias, and excess of Mg can cause absence of reflexes, discomfort, somnolency, among other problems [16].

Iron is found in foods and the main function of this element is the synthesis of hemoglobin, the insufficiency of this mineral can cause anemia that has symptoms as: weakness, dizziness, pallor, loss of appetite, among others [17]. The excess of Fe may cause mainly oxidative stress which would result in damage to cells and tissues [18].

Cr is another metal usually necessary for the proper functioning of the organism, this element is most commonly present in two oxidative states: Cr (III) and Cr (VI). The Cr (III) is stable and its function is to control insulin levels in the body, decreasing the glucose tolerance, while Cr (VI) is highly toxic [19].

Copper is another essential metal to the human organism, this element is mainly found as Cu (II) and is part of enzymes such as: ferroxidase, monoamine, oxidase, tyrozinase, among others. Deficiency of this metal reduces the activity of these enzymes causing anemia, neurological abnormalities and hypopigmentation. Copper chronic intoxication may cause Wilson's disease,

which has symptoms like liver cirrhosis, neurological disorders and kidney damage [20].

In addition to the elements mentioned before, it is possible to find toxic metals such as Cd and Pb in food that may come from both contamination during the industrial process and/or from the raw material. Cd and Pb are two elements considered to be carcinogenic [21]. The major site of action of Pb is the neurological system, but it can also affect the kidneys and the gastrointestinal system. Cd is deposited in the liver and kidneys causing renal, gastrointestinal and pulmonary dysfunctions [7,21]. It can be noted that the macro and micronutrients act beneficially in the human organism, but in excess they compromise the good functioning of this. Therefore, the control of these elements and also contaminants is extremely relevant.

1.2 Determination of essential and trace elements in juice and dietary supplements

Spectrometric methods are widely used for elemental determination in food samples as juices and dietary supplemets. JALBANI et al. [22] determined Cu, Fe and Zn in orange juice by Flame Atomic Absorption Spectrometry (FAAS). The authors also evaluated the efficiency of the sample preparation method using microwave oven acid digestion and compared with the use of acidic digestion using concentrated HNO_3, H_2O_2 and heating in a hot plate. In this study, the two methods presented similar results, however, the use of microwave resulted a higher frequency of sampling and lower consumption of reagents when compared to the conventional method (heating by hot plate). TORMEN et al. [7] determined Ca, Cd, Co, Cu, Fe, Mn, Mo, Na, Ni, Pb, Rb, Sr, V and Zn in orange and peach juice samples using ICP-MS. The authors proposed only dilution with HNO_3 (1% $v.v^{-1}$) as a sample preparation method and compared the results with acid digestion in a microwave oven. The dilution step was considered faster and less prone to contamination than the acid digestion, in addition, the dilution of 20-

fold allowed the direct sample introduction of the equipment, avoiding an obstruction of the nebulization system and deposits in the torch for the analyzed samples [7].

BOUSSAÏDI [23] determined Ca, Mg and Na in samples of water extracted of fresh orange juice using the LIBS technique. The main goal of this study was to evaluate the concentration of these analytes, performing a correlation with the characteristics of cultivation soil. The author analyzed the water and to calculate the final concentration it was applied a external calibration with aqueous standard solutions and the Boltzman equation was used to provide a calibration curve. As expected, the concentration values varied according to region where the plants were cultivated.

KRAWCZYK [24] analyzed commercial dietary supplements and determined As, Cd, Cr, Cu, Fe, Mn, Pb using HR-CS GF AAS (Solid sampling high resolution continuum source furnace atomic absorption spectrometry). The author performed two sample preparation procedures (acid digestion in microwave and suspension analysis). Both methods proved to be effective for the determination of these elements and all presented very low concentrations (μg g^{-1}). WOLLE et al. [5] analyzed 3 samples of children's dietary supplements and 7 of supplements for pregnant women (prenatal supplements). For the sample preparation was used 0.25 g of sample, 5 mL of 0.3 mol L^{-1} ortophosphoric acid (H_3PO_4) and microwave heating. The extracted As was speciated by IC-ICP-MS (ion chromatography-inductively coupled plasma mass spectrometry). The limit of detection (LOD) found for As species was in the range of 2-8 ng g^{-1} and all samples presented arsenite (AsO(OH)) and dimethylarsinic acid ($(CH3)_2As^+(OH)_2$), the arsenate ($AsO(OH)_3$) was found just in two samples [5].

BU et al. [25] determined Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, V, and Zn in seven herbal supplements using a LA-ICP-MS and ICP-MS. For the LA-ICP-MS analysis, 0.5 g of each sample was pelletized and for the ICP-MS analysis an acid digestion was applied using 0.2 g of each sample with 10 mL of

HNO_3, 1 mL of H_2O_2 and 1 mL of HF. For the external calibration were used pellets of some SRM (Standard Reference Material) and aqueous standard solutions for the LA-ICP-MS and ICP-MS analysis, respectively. The authors obtained good recoveries and concentration values similar for the both techniques showing that the LA-ICP-MS can be a suitable alternative for these type of samples [25].

It was possible to notice that for almost all analysis a sample preparation step is required. A sample preparation process can show a lot of challenges and problems such as contaminations, loss of analytes and in almost the cases a large consumption of reagents and samples, not contributing to green chemistry [26]. In this context, analytical techniques in which the need for sample preparation is minimal or nonexistent become a major attraction for analytical analysis (quantitative and qualitative).

1.3 LIBS and Laser Ablation

Techniques based on ablation using laser pulses deserve special mention when a sample preparation process needs to be avoided or minimized. Some techniques that use laser sources are: LIBS, LIF (Laser-induced fluorescence), and LA-ICP-MS.

The laser principles are based on the stimulated emission process. In this process the excitated specie is reached by photons with the same energy that the photons produced by spontaneous emission, all this process cause a population inversion of photons and production of energy. FIGURE 1 shows a schematic reproduction of the stimulated emission process and laser system.

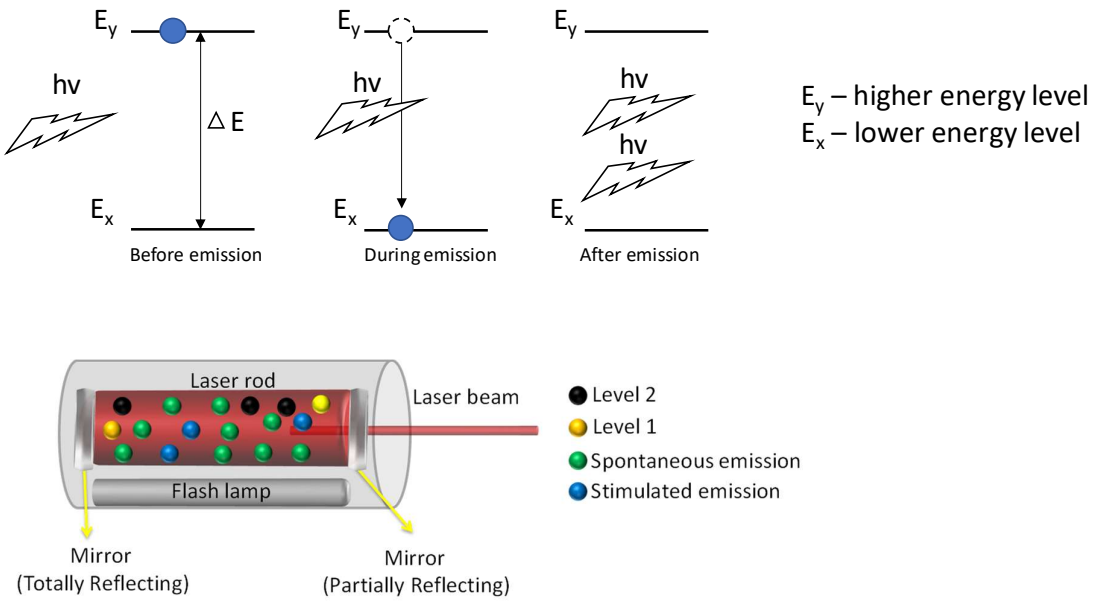

FIGURE 1 Stimulated emission process and laser system**.**

In the techniques with laser sources as a LIBS system (FIGURE 2) the laser pulse beam is focused on the surface of the sample, the radiation energy is located at micrometric points on the sample surface that loses matter and begins the formation of a plasma, this plasma decays emitting a specific radiation for each element [27].

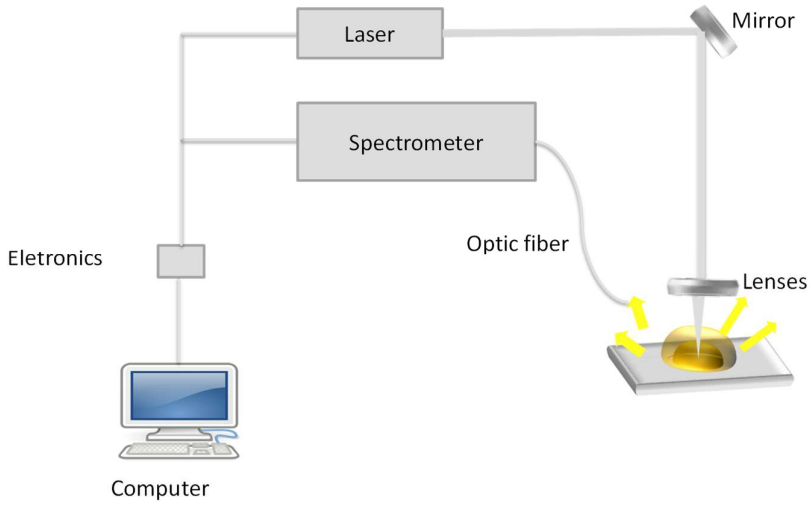

FIGURE 2 Schematic representation of a typical LIBS system.

LIBS is a multielement technique and show advantages such as: speed, versatile and possibility of direct analysis of solid, liquid and gaseous samples, with the ablated masses in the order of picograms to nanograms, also the samples can be analyzed with or without a minimum sample preparation [28]. In quantitative analysis, as in all other methods, a calibration procedure is required. Few possibilities of calibration can be applied to direct analysis, options as external calibration (with reference material or in-house fabricated standards), multivariate calibration, internal standards and calibration free method (CF-LIBS) can be one of these possibilities [28]. Despite the great advantages cited, the LIBS presents some difficulties as the strong matrix effects and spectral interferences and high LOD and LOQ depending on the instrument set up and type of samples [29]. For techniques as LA-ICP OES and LA-ICP-MS the process of ablation is similar but in these cases the measuared is from the sample particles that is carried for ICP plasma.

According to the literature, the studies using LIBS for quantitative purposes in samples of juices and food supplements are minimum or nonexistent. Thus the following discussion will be performed with studies using other matrices. LEME et al. [30] determined Ca, K, Mg and Na in bovine and chicken meat samples using a commercial system LIBS. The authors made their own standards for the LIBS analysis, to achieve these standards was proposed two different procedures: (i) the dilution of the bovine and chicken meat (previously dried in a freezer-dryer and ground in a cryogenic mill) with cellulose binder in proportions of 25 and 50 % (w w^{-1}) and (ii) spiking 3.5 g of milled meat with 20 mL of stock solutions of the analytes, the standards were frozen with liquid N_2, homogenized in a cryogenic mill and pelletized. Certified reference material (CRM) was used to verify the accuracy, the concentration values obtained by LIBS was also compared with the results obtained by ICP OES. The accuracy values were satisfactory and no significant differences was found between the LIBS and ICP OES results.

BILGE et al. [31] developed a method to detect and quantify adulterations of powder milk with whey powder using LIBS. Principal component analysis (PCA) was used to discriminate the samples of powder milk and the powder milk with whey powder, the discrimination ratio of the 21 samples was 80.5%. An external calibration was applied with in-house fabricated standards, a PLS regression model was calculated and the correlation coefficient (R^2) and LOD values were 0.981 and 1.55% (adulteration with sweet whey powder) and 0.985 and 0.55% (adulteration with acid whey powder), respectively.

The LOD of LIBS are of the order of mg kg^{-1} [29], and can increase considerably when the sample analyzed is in the liquid state. Problems such as decreased plasma intensity and bubble formation are just a few of the obstacles to these types of analysis. An alternative method that can be associated with liquid analysis is the preconcentration processes thus increasing sensitivity and decreasing the limits of detection [32].

AGUIRRE et al. [32] resolved this disadvantage of liquid analysis via LIBS using the liquid-liquid microextraction method and analyzing the dry microdroplets on metal supports. In the experimental procedure, aqueous solutions containing Mn were analyzed and the method presented greater sensitivity, precision and linearity of the curve when compared to the direct analyzes of the droplets. JESUS et al. [33] determined Mo and V in several matrices (drugs and soils) combining liquid-liquid microextraction and LIBS. The LOD for these elements were the order of 30 and 5 μg kg^{-1} for Mo and V, respectively.

SEZER et al. [34] used as strategy to transform liquid samples of milk into gels and avoid problems with liquid analysis by LIBS. Thirteen bovine, fourteen ovine and thirteen caprine milk samples were dissolved in gelatin to produce a solid sample. The pure samples (caprine, bovine and ovine) and samples of caprine and ovine milk adulterated with bovine milk were analyzed by LIBS, a PCA and PLSR were calculated. The values of correlation coefficient and

LOD were 0.995 and 1.39% (caprine milk with bovine milk as adulterant) and 0.996 and 1.29% (ovine milk with bovine milk as adulterant), respectively.

1.4 Chemometrics

Chemometrics was developed mainly in the 1970s along with the establishment of scientific computation and involved in particular the statistical treatment of analytical chemistry data.

In any chemical analysis it is necessary to find a better working condition that will bring the best response. The biggest challenge in achieving a better response in an experiment is to identify the influence (effect) of some factors on the response. When using the method of setting a factor (variable) and varying the others until a better answer is found, this is probably not really the best, unless all the variables are totally independent (knowledge that the analyst does not previously have) [35].

In most chemical experiments, the variables are not independent of each other (they present interaction). In these cases, to evaluate the influence of all the factors on the response and to vary all of them at the same time with a minimum number of experiments, is used of design of experiments [36].

When it is desired to identify similarities and differences among analyzed samples, it is possible to use chemometric tools to treat the obtained data. One of these tools is the exploratory analysis of data using Principal Component Analysis (PCA). Using PCA, it is possible to obtain a reduction of the original dimension of the data through manipulation of the data matrix, where they are represented in new coordinates called Principal Components (PC). With PCA it is also possible to model, detect anomalous samples and establish an unsupervised classification of them [37].

For the calibration processes PCR (Principal Component Regression) and PLS can be used as chemometric strategies. In the PCR only the instrumental responses are taken while in the PLS also the concentrations are taken into account

[38]. Both methods are based on data obtained by a PCA and all its advantages can be combined using hybrid PCR and PLS models to reach more accurate models [39]. Unlike PCR and PLS there is a technique called multiple linear regression (MLR). In this method all data matrix **X** (matrix with intensity signals for example) is used including irrelevant information, but a major problem presented by this technique is collinearity, requiring more samples than variables [38].

DOUCET et al. [40] used the multivariate calibration method, MLR for the determination of macro and microelements present in Al alloys. The analytes were determined by LIBS and the combination of the MLR with a non-linear pretreatment showed accuracy better than 5% for the major elementsg of the metal alloys. MUKHONO et al. [41] used the LIBS technique together with chemometric tools to characterize environmental samples (e.g. rocks and soils). The authors proposed calibration models to quantify As, Cr, Cu, Pb and Ti, using PLS and ANNs (Artificial Neural Networks). PLS presented better models for soil samples while ANNs were more suitable for rock samples. For a qualitative and exploratory analysis of the data were used PCA and SIMCA (Soft Independent Modelling of Class Analogy).

ABDEL-SALAM et al. [42] analyzed samples of breast milk during the first 3 months of lactation and compared these samples with 6 different types of commercial infant formula. The authors evaluated the levels of Ca, Fe, Mg and Na using the spectral emission lines generated by LIBS spectrum. Breast milk samples showed much higher levels of Ca, Fe, Mg and Na when compared to commercial formulas.

Although all the papers presented are still few, they refer to food analysis using LIBS technique with or without the union with chemometrics.

Chapter 2 – Goals

2. Goals

The main goal of this PhD thesis was the development of analytical strategies for the determination of Ca, Cd, Cr, Cu, Fe, Mg, Pb and Zn in orange juice and dietary supplements by laser techniques.

2.1 Specific Goals

1. To develop strategies for the direct and quantitative analysis of nutrients and contaminants in dietary supplements and powder milk (Ca, Cd, Cr, Cu, Fe, Mg, Pb and Zn) using LIBS, LA-ICP-MS and LA-ICP OES;

2. To develop strategies for determination of Ca, Cd, Cr, Cu, Fe, Mg, Pb and Zn in liquid samples, orange juice, by LIBS and LA-ICP-MS;

3. Explore differents calibration strategies for quantitative analysis of dietary supplements (LIBS, LA-ICP OES and LA-ICP-MS) and powder milk (LIBS);

5. Explore the possibilities to improve figures of merit, such as LOD and RSD, for the quantitative analysis of liquid sample, by LIBS and LA-ICP-MS;

6. Apply the DLLME as an alternative to improve the limit of quantification values.

Chapter 3 – References

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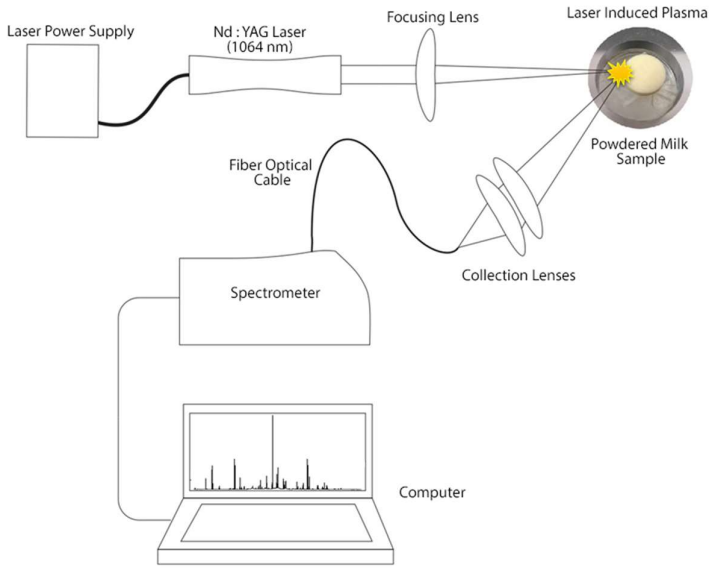
Chapter 4 – Published Results

4. Published Results

4.1 "Calibration strategies for the direct determination of Ca, K, and Mg in commercial samples of powdered milk and solid dietary supplements using laser-induced breakdown spectroscopy (LIBS)" *Food Research International* 94 (2017) 72–78.

Graphical Abstract



Food Research International 94 (2017) 72–78

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Calibration strategies for the direct determination of Ca, K, and Mg in commercial samples of powdered milk and solid dietary supplements using laser-induced breakdown spectroscopy (LIBS)

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ARTICLE INFO

Article history:
Received 26 November 2016
Received in revised form 23 January 2017
Accepted 28 January 2017
Available online 31 January 2017

Keywords:
Laser-induced breakdown spectroscopy
Powdered milk
Dietary supplements
Direct solid analysis
Chemometrics
Calibration

ABSTRACT

This study describes the application of laser-induced breakdown spectroscopy (LIBS) for the direct determination of Ca, K and Mg in powdered milk and solid dietary supplements. The following two calibration strategies were applied: (i) use of the samples to calculate calibration models (milk) and (ii) use of sample mixtures (supplements) to obtain a calibration curve. In both cases, reference values obtained from inductively coupled plasma optical emission spectroscopy (ICP OES) after acid digestion were used. The emission line selection from LIBS spectra was accomplished by analysing the regression coefficients of partial least squares (PLS) regression models, and wavelengths of 534.947, 766.490 and 285.213 nm were chosen for Ca, K and Mg, respectively. In the case of the determination of Ca in supplements, it was necessary to perform a dilution (10-fold) of the standards and samples to minimize matrix interference. The average accuracy for powdered milk ranged from 60% to 168% for Ca, 77% to 152% for K and 76% to 131% for Mg. In the case of dietary supplements, standard error of prediction (SEP) varied from 295 (Mg) to 3782 mg kg^{-1} (Ca). The proposed method presented an analytical frequency of around 60 samples per hour and the step of sample manipulation was drastically reduced, with no generation of toxic chemical residues.

1. Introduction

An equilibrated and healthy diet includes a large portion of chemical components essential for the proper physiological functions of the human body. Mineral elements, as Ca, K, Mg, and Na (macro elements), Cr, Cu, Fe, Mn, Se, and Zn (micro and trace elements) are generally found in a wide range of foods (Belitz, Grosch, & Schieberle, 2009). Calcium is involved in several processes, such as blood coagulation, muscular contraction and bone formation (Allgrove, 2003). Magnesium is a cofactor of enzymatic reactions (Barbagallo & Dominguez, 2007), and K participates in intracellular osmolality (Ekmekcioglu, Elmadfa, Meyer, & Moeslinger, 2016). A daily dose of these elements is important for the human body. On the other hand, this does not always occur, then fortified foods as powdered milk and solid dietary supplements can become an option, due to the special needs of some people, such as pregnancy and children with a restrictive diet. Powdered milk and solid dietary supplements contain a range of essential elements responsible for the health and child growth, being necessary a fast and reliable analytical method for quality control.

Traditional analytical techniques, such as inductively coupled plasma optical emission spectrometry (ICP OES), flame atomic absorption spectrometry (FAAS) and ICP-mass spectrometry (ICP-MS) have been applied for the quantification of several elements, but generally require a pre-treatment to convert the solid sample to an aqueous homogeneous solution. During the analytical sequence, errors can be introduced due to the manipulation that reduces analytical frequency and generate residues (Chinni, Cremers, & Multari, 2010). Other analytical strategies include analysis of samples as suspension using ICP OES or FAAS (Asfaw & Wibetoe, 2005; Sola-Larrañaga & Navarro-Blasco, 2009).

Analytical techniques such as laser-induced breakdown spectroscopy (LIBS) has been used in several applications related to food samples, like classification of red wine (Moncayo, Rosales, Izquierdo-Hornillos, Anzano, & Caceres, 2016), identification of meat species (Bilge, Velioglu, Sezer, Eseller, & Boyaci, 2016), and boron determination in

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http://dx.doi.org/10.1016/j.foodres.2017.01.027
0963-9969/

meatballs (Hedwig et al., 2016). For those studies, LIBS advantages include: direct analysis with minimal or no sample preparation, high analytical frequency, the use of a small quantities of sample (typically <100 mg), and multielement capability (advantage if compared with FAAS) (Pasquini, Cortez, Silva, & Gonzaga, 2007). Spectra obtained from LIBS technique present several emission lines allowing a fast sample inspection and the application of chemometrics tools for calibration and classification purposes (Neiva, Chagas Jacinto, Mello de Alencar, Esteves, & Pereira-Filho, 2016). For calibration, several regression models, employing univariate or even, multivariate analysis are possible, using for instance partial least squares (PLS) (El Haddad, Canioni, & Bousquet, 2014; Hernández-García et al., 2017).

On the other hand, disadvantages related to method calibration are observed, because the ablation process involves some μg of samples and it is not available reference material with certified values concentration for masses in the range of μg or ng, for example (Andrade & Pereira-Filho, 2016; Andrade, Pereira-Filho, & Konieczynski, 2016; Augusto, Sperança, & Andrade, 2016). In addition, direct analysis presented some difficulties in solid analysis, such as the reproduction of the data related to the process of ablation, formation of the plasma, microheterogeneity and matrix effects. These issues can compromise quantitative analysis (Mukhono, Angeyo, Dehayem-Kamadjeu, & Kaduki, 2013).

The goal of this study is to present a simple and fast method for the direct analysis of powdered samples of milk and solid dietary supplements using LIBS and determine the contents of three major constituents: Ca, Mg and K. Univariate and multivariate analysis strategies were tested and combined to build regression models. Some procedures, for instance sample dilution, were used to minimize matrix effects and achieve results with satisfactory accuracy, when solid samples are directly measured.

2. Materials and methods

2.1. Reagents, sample description and preparation for ICP OES determinations

All reagents were of analytical grade or higher purity. Deionized water (18.2 ΩM cm resistivity) produced by a Milli-Q® Plus Total Water System (Millipore Corp., Bedford, MA, USA) was used to prepare all of the solutions. Prior to use, all glassware and polypropylene flasks were washed with soap, soaked in 10% v/v HNO_3 for 24 h, rinsed with deionized water and dried to ensure that no contamination occurred. The multi-element standard solutions were prepared daily from 10,000 mg L^{-1} Ca along with 1000 mg L^{-1} K and Mg stock solutions (Qhemis, Jundiaí, SP, Brazil) and used for construction of the calibration curve.

Powdered samples of milk (M) and solid dietary supplements (S) were purchased at the local markets of São Carlos (São Paulo State, Brazil). Fifteen powdered milk samples (M1–M15), intended to be consumed by adults and children, and 8 samples of solid dietary supplements (S1–S8), intended only for children, were analysed without any prior treatment by LIBS. The selected samples were from different manufacturers and flavours (case of dietary supplements) to introduce a high sample variability in the calibration models proposed. In the case of powdered milk, skimmed, whole and with vegetable oils samples were selected. To establish regression models, concentrations of Ca, K and Mg, obtained with ICP OES (iCAP 6000, Thermo Scientific, Waltham, MA, USA), were used as reference values ($n = 3$), after acid digestion for mineralization of the samples. This instrument allows sequential analytical signal collection using both axial and radial views. Argon (99.996%, White Martins-Praxair, Sertãozinho, SP, Brazil) was used for all ICP OES measurements. ICP OES operational parameters and the emission lines used are shown in Table 1.

A microwave system (Speedwave Four, Berghof, Eningen, Germany) was employed to mineralize the samples for further ICP OES determinations. The microwave system was equipped with twelve high pressure TFM (a copolymer of tetrafluoroethylene and a small amount of the perfluoro(propyl vinyl ether)) vessels (100 mL, 230 °C and 70 bar), and the microwave heating program is shown in Table 2. In the case of powdered milk samples, 250 mg were mixed with 6 mL of HNO_3 (2 mol L^{-1}) (Synth, Diadema, SP, Brazil) and 3 mL of H_2O_2 (30% w/w) (Synth). This digestion mixture was already proposed by Bizzi et al. (2014). For solid dietary supplements only 6 mL of HNO_3 (2 mol L^{-1}) was employed and in both cases, no residues were observed in the resultant digested solution. The concentrated HNO_3 was previously purified using a sub-boiling distillation system Distillacid™ BSB-939-IR (Berghof, Eningen, Germany). After digestion, the final volume was adjusted to 14 mL with deionized water.

Table 1
ICP OES instrumental conditions to obtain reference values for Ca, K and Mg.

Parameters	Operational conditions
Integration time for low emission line (s)	5
Integration time for high emission line (s)	5
Sample introduction flow rate (mL min^{-1})	2.1
Sample flow rate during the analyses (mL min^{-1})	2.1
Pump stabilization time (s)	25
Radio frequency applied power (W)	1200
Auxiliary gas flow rate (L min^{-1})	0.25
Nebulization gas flow rate (L min^{-1})	0.83
Cooling gas flow rate (L min^{-1})	16
Lines for Ca, K and Mg on axial and radial view (nm)	Ca (II 317.9), K (I 691.1, I 766.4 and I 769.8) and Mg (II 279.5, II 280.2 and I 285.2)

I: Atomic.
II: Ionic.

2.2. LIBS system

A LIBS system (model J200, Applied Spectra, Fremont, California, USA) with Axiom 2.5 software was used to detect the emission lines of Ca, K and Mg in powdered samples of milk and solid dietary supplements. This instrument is equipped with an ablation chamber and a HEPA air cleaner to purge ablated solid particles. The laser (Nd:YAG) was operated at a fundamental wavelength of 1064 nm, and the maximum energy is 100 mJ in a single laser pulse with an 8 ns duration at a frequency of 10 Hz.

The plasma radiation emission was directly collected by an optical fibre coupled to a 6-channel CCD spectrometer with a spectral window ranging from 186 to 1042 nm. The spectral resolution varies from <0.1 nm in the ultraviolet to visible (UV–Vis) range up to 0.12 nm in the UV and near infrared (NIR) range. In the identification of the emission lines, Aurora software (also from Applied Spectra) was used. As solid samples without any sample preparation were used and in order to perform the measurements, 500 mg pellets were pressed at 10 tons using a hydraulic press. The pellets were introduced in the LIBS system and from 9 to 16 straight line scans, each with 9 mm of length, were applied, and the distance between the lines was 1 mm. Approximately 1000 spectra were recorded for each sample. The operational LIBS data collection parameters were previously optimized using a factorial

Table 2
Microwave heating program applied for sample mineralization.

Step	Power (W)	Temperature (°C)	Ramp time (min)	Hold time (min)
1	1260	120	5	5
2	1260	160	5	5
3	1260	230	5	10

Table 3
Solid calibration mixtures of solid dietary supplements (S) used in the univariate linear models for Ca, K and Mg.

Calibration solid mixture identification	Weight of each sample (g)					Analytes concentration (mg kg^{-1})		
	MC[a]	Mass1	Mass2	Mass3	Mass4	Ca	K	Mg
Blank	4.000	0.000	0.000	0.000	0.000	0	0	0
Standard 1	2.790	0.000	0.723	0.480	0.000	3900	2195	426
Standard 2	0.147	0.885	0.848	0.519	1.615	6330	7942	857
Standard 3	0.115	0.334	2.353	0.752	0.461	10,980	8060	1169
Standard 4	0.000	0.000	4.000	0.000	0.000	14,067	9128	1229
Standard 5	0.000	4.004	0.000	0.000	0.000	2890	10,318	1006
Standard 6	0.000	0.000	0.000	4.018	0.000	11,340	5720	1708
Standard 7	0.000	0.000	0.000	0.000	4.015	3121	2637	385

[a] Microcrystalline cellulose.

design, and the values selected were a delay time of 0.5 μs, a spot size of 50 μm, an energy of 50 mJ, a scan speed of 1 mm s^{-1} and a laser repetition rate of 10 Hz. Three pellets were prepared for each sample ($n = 3$) in order to evaluate the errors of the proposed method.

2.3. Calibration strategies

As previously mentioned, calibration using solid samples is a challenging task for any analytical method that performs direct measurements. In this case, two calibration strategies were investigated and implemented in this study.

To overcome some drawbacks of LIBS, the normalization of the data and instrumental parameters optimization (Klus et al., 2016; Pořízka et al., 2016) can be performed. In this sense, 12 types of normalization modes (Castro & Pereira-Filho, 2016) were tested in both cases (milk and solid dietary supplement). The goal of the normalization is to minimize problems related to sample microheterogeneity and signal fluctuation during data acquisition. In this sense, normalization by norm (Euclidean vector), signal area and height and carbon emission lines (used as internal standard) were evaluated for each type of sample.

For data set organization, Microsoft Excel™ was used. A critical step in the calibration is the selection of the most appropriate emission lines (free of spectral interference) to perform the calculations. A strategy used was in a first step, calculate PLS models using the entire peak profile (12,288 variables, from 186 to 1042 nm), the data set was mean-centred, and cross validation (leave-one-out, one by one sample) was used to identify the proper number of latent variables (LV). Reference concentrations obtained after microwave digestion and ICP OES determinations, were used as dependent variables (matrix Y). After inspecting the PLS regression vectors, those emission lines for Ca, K and Mg with high values and/or free of spectral interference were selected to calculate further univariate models using both signals, area and height.

Matlab (MathWorks, Natick, Massachusetts, USA) version 2010, was used for the normalization of the spectra and, both signals - area and height calculation, for the selected emission lines, and Pirouette Multivariate Data Analysis software, version 4.5 (Infometrix, Bothell, WA, USA), was used to calculate the PLS calibration models.

In the case of powdered milk, the 15 samples were pressed (10 tons in.$^{-1}$, 12 mm diameter) and 10 samples were applied to calculate univariate calibration models (Ca, K and Mg). The obtained models were tested on the remaining 5 samples.

For the solid dietary supplements, a calibration curve with the same matrix as the samples was constructed. In this case, microcrystalline cellulose (P.A., Synth) was used as a blank and mixed with different samples amount to build a calibration curve. This calibration curve was organized by mixing different proportions of the samples targeting a wide range of concentrations for the analytes under investigation. A calibration curve with 8 points was prepared by mixing 4 samples that presented the highest and lowest concentration values for Ca, K and Mg. The proportions of the samples in the mixture for each point and the final analytes concentration are shown in Table 3.

Only 500 mg of each mixture was used to prepare the pellets and 3 replicates were organized for each standard. The use of mixtures of

Table 4
Reference (ICP OES, $n = 3$), determined values (LIBS, $n = 3$), and accuracy for powered milk (M) and solid dietary supplements (S) (concentrations in mg kg^{-1}).

Sample ID	Ca			K			Mg		
	Reference concentration (ICP OES)	Determined concentration (LIBS), I 534.945 nm	Accuracy (%)	Reference concentration (ICP OES)	Determined concentration (LIBS), I 766.490 nm	Accuracy (%)	Reference concentration (ICP OES)	Determined concentration (LIBS), I 285.235 nm	Accuracy (%)
Powdered milk (M), calibration dataset									
M2	4762 ± 136	4493 ± 573	94	7266 ± 1682	6309 ± 422	87	449 ± 10	480 ± 20	107
M3	2759 ± 142	4647 ± 564	168	4227 ± 1173	6438 ± 657	152	545 ± 61	582 ± 38	107
M4	13,482 ± 1100	17,298 ± 1001	128	13,707 ± 516	16,831 ± 2026	123	950 ± 75	963 ± 95	101
M5	4289 ± 117	4414 ± 478	103	6724 ± 1571	6206 ± 427	92	423 ± 9	457 ± 13	108
M6	24,178 ± 2494	23,721 ± 1326	98	14,148 ± 1753	13,305 ± 1170	94	1167 ± 61	1205 ± 59	103
M7	2586 ± 397	1547 ± 357	60	4113 ± 33	4104 ± 703	100	286 ± 69	265 ± 21	92
M9	5164 ± 549	5481 ± 362	106	5976 ± 187	5931 ± 684	99	391 ± 78	402 ± 12	103
M10	2970 ± 442	2575 ± 1230	87	4167 ± 58	3789 ± 522	91	415 ± 90	317 ± 37	76
M12	8880 ± 912	7835 ± 674	88	10,876 ± 1164	8370 ± 660	77	766 ± 54	619 ± 74	81
M14	11,345 ± 458	8404 ± 942	74	8473 ± 1537	8394 ± 1791	99	480 ± 13	582 ± 73	121
Powdered milk (M), validation dataset									
M1	13,032 ± 441	11,287 ± 2051	87	8727 ± 492	7931 ± 675	91	637 ± 24	667 ± 71	105
M8	4788 ± 520	4911 ± 1555	103	5969 ± 28	5034 ± 1209	84	375 ± 75	329 ± 43	88
M11	8997 ± 303	8849 ± 326	98	7086 ± 655	9480 ± 1326	134	495 ± 53	649 ± 25	131
M13	6787 ± 983	4202 ± 605	62	6548 ± 162	5965 ± 411	91	434 ± 72	369 ± 42	85
M15	12,480 ± 1349	8373 ± 732	67	7002 ± 260	7610 ± 600	109	487 ± 65	459 ± 30	94
Dietary supplement (S), prediction dataset									
S1	12,889 ± 206	5300 ± 1173	41	7581 ± 188	5351 ± 239	71	773 ± 17	487 ± 35	63
S2	4374 ± 377	2584 ± 511	60	10,318 ± 270	8436 ± 330	82	693 ± 63	291 ± 12	42
S3	2887 ± 119	1741 ± 521	60	2691 ± 279	1539 ± 485	57	1006 ± 59	778 ± 101	77
S4	10,319 ± 799	6575 ± 1537	64	5721 ± 101	10,041 ± 424	176	1451 ± 225	1546 ± 82	107
S5	11,340 ± 332	10,687 ± 1140	94	2638 ± 68	1664 ± 395	63	1708 ± 160	1655 ± 48	97
S6	3121 ± 125	4807 ± 630	154	3857 ± 145	6721 ± 344	174	385 ± 5	396 ± 13	103
S7	4267 ± 281	3615 ± 730	85	3566 ± 88	2352 ± 985	66	1031 ± 83	986 ± 397	96
S8	14,067 ± 506	9561 ± 1294	68	9128 ± 208	11,359 ± 1134	124	1229 ± 109	681 ± 22	55

samples to generate a calibration curve was a strategy to minimize interference from sample matrix under analysis (Gilon et al., 2011; Lei et al., 2011). Univariate models were established with these 8 calibration points (Table 3), and the predictive ability of the models was tested using 8 external samples (samples S1–S8, prediction dataset).

3. Results and discussion

3.1. ICP OES reference analytical method

As mentioned before, a digestion of all the samples (powered milk and solid dietary supplements) was performed and the solutions were analysed by ICP OES to obtain reference values for Ca, K and Mg. Table 1 presents the emission lines studied in ICP OES instrument in axial and radial views. The emission lines that presented concordant results between axial e radial views were selected and the average of the concentrations ($n = 3$) results was considered as reference values. Table 4 shows the reference values for these elements in powered milk (M) and solid dietary supplements (S).

The selected lines in ICP OES measurements were different for the powered milk and solid dietary supplements. To the mineralized milk were selected the lines: Ca II 317.9 nm, K I 691.1 nm and Mg II 279.5 nm. To the mineralized dietary supplements were selected the lines: Ca II 317.9 nm, K I 766.4 nm and I 769.8 nm and Mg II 279.5 nm, II 280.2 nm and I 285.2 nm.

3.2. Emission line selection in LIBS calibration and powdered milk analysis

To select the emission lines that present signals linearly proportional to the concentrations; firstly, PLS regression vectors were verified. In this evaluation, emission lines free of interferences for Ca, K and Mg were selected. More than 10 emission lines were investigated for each analyte. Fig. 1 shows the regression coefficients for Ca (Fig. 1a), K (Fig. 1b) and Mg (Fig. 1c). For each multivariate calibration models, the selected number of latent variables (LV) were 2, 1 and 2 for Ca, K and Mg, respectively.

Several emission lines were identified with the help of PLS regression coefficients and by means of these individual emission lines from LIBS data, univariate models were calculated using both types of signal information, i.e., the area and height. The lowest standard error of calibration (SEC) was the criteria to select the best lines to comprise the models (Pereira, Pereira-Filho, & Bueno, 2006). SEC values were calculated according to Eq. (1):

$$\mathrm{SEC} = \sqrt{\frac{(y_i - \hat{y}_i)^2}{n-1}} \tag{1}$$

where n is the number of samples and y_i and $\hat{y}_i$ are the reference (ICP OES) and the predicted analytes concentrations, respectively for data set of calibration.

Fig. 2 shows the emission lines selected for Ca I 534.947, K I 766.490 and Mg I 285.213 nm being atomic lines for all, denoted as (I).

The best results were obtained after signal normalization by individual norm and averaged (for Ca), only averaged (for K) and normalized by C I 247.856 nm and later the sum was calculated (for Mg). In the normalization by norm each spectrum was divided by its individual norm (||b||), see Eq. (2):

$$||b|| = \sqrt{signal_1^2 + signal_2^2 + \ldots + signal_n^2} \tag{2}$$

where $signal_n$ is the signal intensity for each emission line (from 186 to 1042 nm). After normalization by norm, each normalized spectrum has norm equals to 1, it means that all spectra have the same size. In the case of normalization by C I 247.856, each signal intensity is divided by C I 247.856 intensity. Later the C I 247.856 signal is equals to 1. After this first step the sum of spectra was calculated. Signals area and height were calculated after baseline offset correction and the proposed univariate models presented R^2 values ranging from 0.7965 (K) to 0.9216 (Ca) when area was considered.

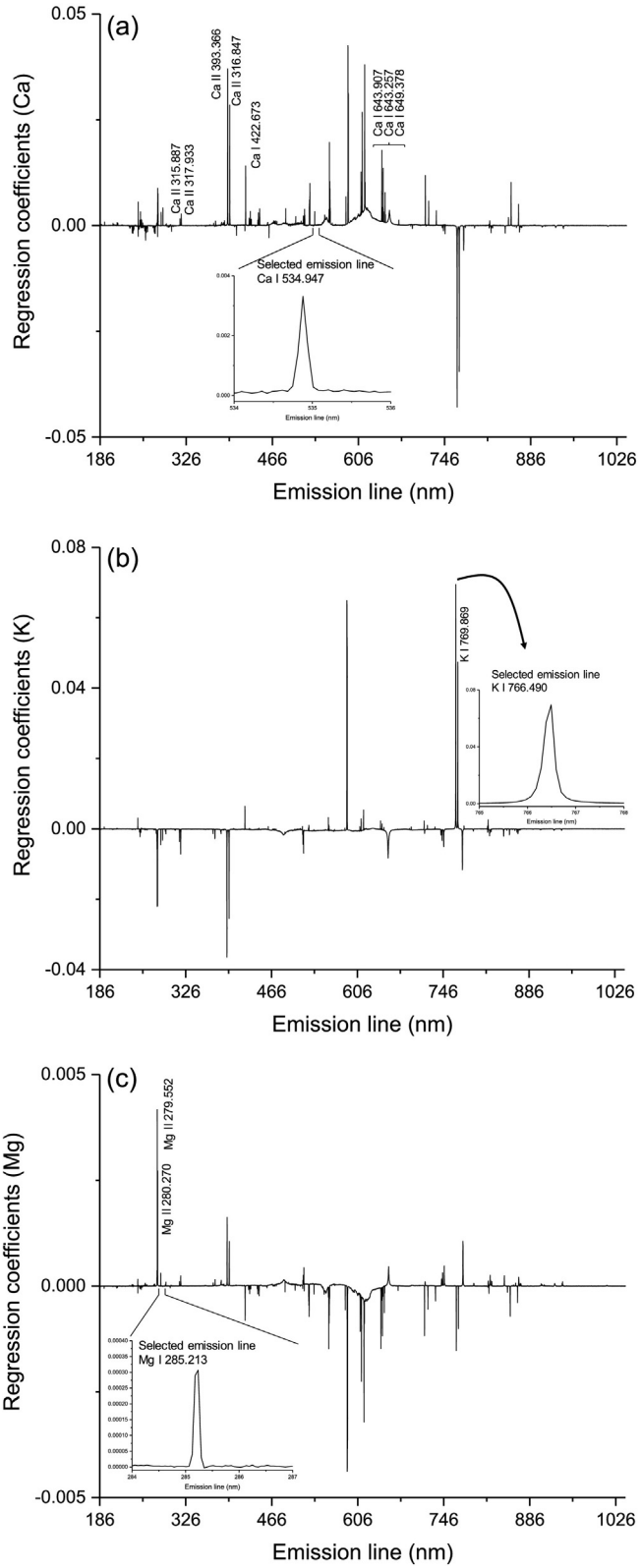

Fig. 1. Regression coefficients for PLS models calculated for Ca (a), K (b) and Mg (c).

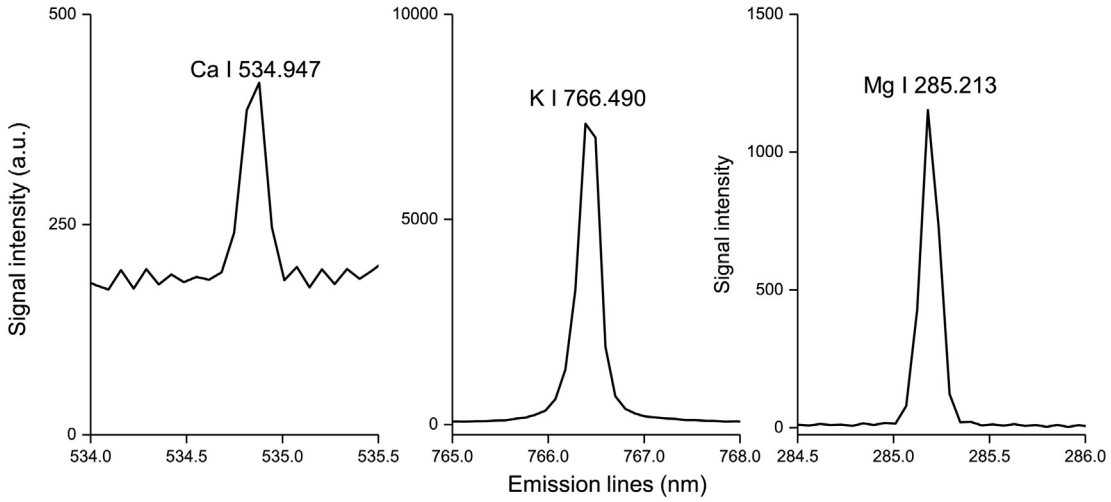

Fig. 2. Emission lines selected for Ca, K and Mg in LIBS univariate models.

The SEC values for Ca, K and Mg were 1895, 1825 and 85 mg kg^{-1}, respectively. In addition, all analytes presented good correlation, when reference and predicted values were compared, with R^2 values varying from 0.2681 (K in validation dataset) to 0.9616 (Ca in calibration dataset). The predicted concentrations and its standard deviation ($n = 3$) are presented in Table 4 and the analytical parameters are shown in Table 5.

The other normalizations presented approximately 5-fold higher SEC values. Surprisingly, normalizations using C emission lines presented suitable SEC value only for Mg, considering the content of C is higher than 90% in this type of samples. This calibration model can be used in the quality control of this type of sample. Fig. 3 shows the reference and predicted concentrations for milk samples for calibration (circles) and validation (squares) datasets. As 3 replicates were prepared average RSD values were 15% for Ca, 12% for K and 8% for Mg. Accuracy was calculated using Eq. (3):

$$\text{Accuracy} = \frac{\hat{y}_i}{y_i} \times 100 \quad (3)$$

where y_i and $\hat{y}_i$ are the reference (ICP OES) and the predicted analyte concentration, respectively.

Sample M2, for instance (see Table 4), presented a Ca reference (ICP OES) and predicted (LIBS) concentration of 4762 ± 136 and 4493 ± 573 mg kg^{-1}, respectively. The accuracy for this samples was 94% ($\frac{4493}{4762} \times 100 = 94\%$).

3.3. Analysis of solid dietary supplement samples

The same emission lines described for powdered milk were used for the solid dietary supplements. However, the strategy used in this part was to establish a calibration curve with external standards prepared by mixing different samples in different proportions with microcrystalline cellulose (see details in Table 3).

Initially, the mixtures described in Table 3 were used, and the concentrations varied from 0 (pure cellulose) to 14,067 for Ca, 0 to 10,318 for K and 0 to 1708 mg kg^{-1} for Mg.

In the case of Ca, several problems related with interference were observed, and the calibration ability of the model was highly compromised in all 12 normalization modes. To overcome this issue, dilution of the samples was performed (Jantzi et al., 2016). All pellets (mixtures for calibration, Table 3 and samples) were diluted 10-fold with microcrystalline cellulose, and the Ca concentration in the standards ranged from 0 to 1407 mg kg^{-1}. This approach was tested to reduce the influence of the matrix on the measurements and improve the predictive ability of the proposed calibration models.

Univariate calibration (linear equation) was calculated for Ca, Mg and K and the normalization modes were tested again. For Ca and Mg normalization by norm presented the best results. In the case of K only average present good results. The univariate models using signal area presented R^2 values from 0.8639 for Mg to 0.9102 for Ca. These univariate models were tested in the 8 samples ($n = 3$) and the predicted values can be shown in Table 4. The RSD values average ranged from 10% for Mg to 19% for Ca. The accuracy of the measurements for Ca varied from 41 to 154%, and the R^2 obtained when reference (ICP OES

Table 5
Analytical parameters for powered milk and solid dietary supplements analysed by LIBS.

Sample dataset	Analyte	SEC (mg kg^{-1})	SEV, powdered milk or SEP, dietary supplement (mg kg^{-1})	R^2 (univariate regression model)	R^2 (reference × predicted)	
					Calibration	Validation
Powdered milk	Ca[a]	1895	2614	0.9216	0.9216	0.6336
	K[b]	1825	1548	0.7965	0.7965	0.2681
	Mg[c]	85	91	0.9087	0.9087	0.6770
Solid dietary supplements	Ca[a]	2137	3782	0.9102	–	0.6394
	K[b]	2027	2510	0.8665	–	0.5804
	Mg[c]	357	295	0.8639	–	0.8172

[a] 534.945 nm (I, atomic line).
[b] 766.490 nm (I, atomic line).
[c] 285.235 nm (I, atomic line).

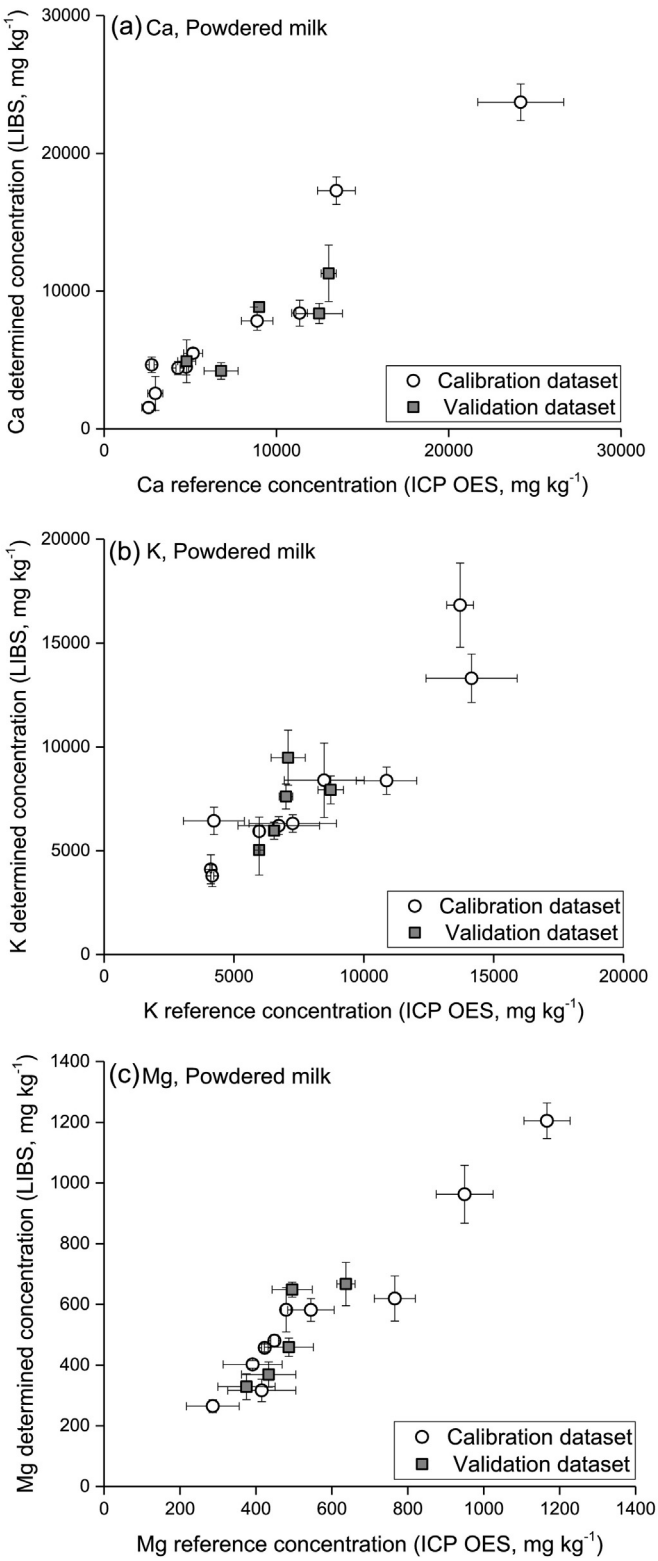

Fig. 3. Reference (ICP OES) and predicted (LIBS) concentrations (mg kg^{-1}) for powdered milk analysis for Ca (a), K (b) and Mg (c) determination.

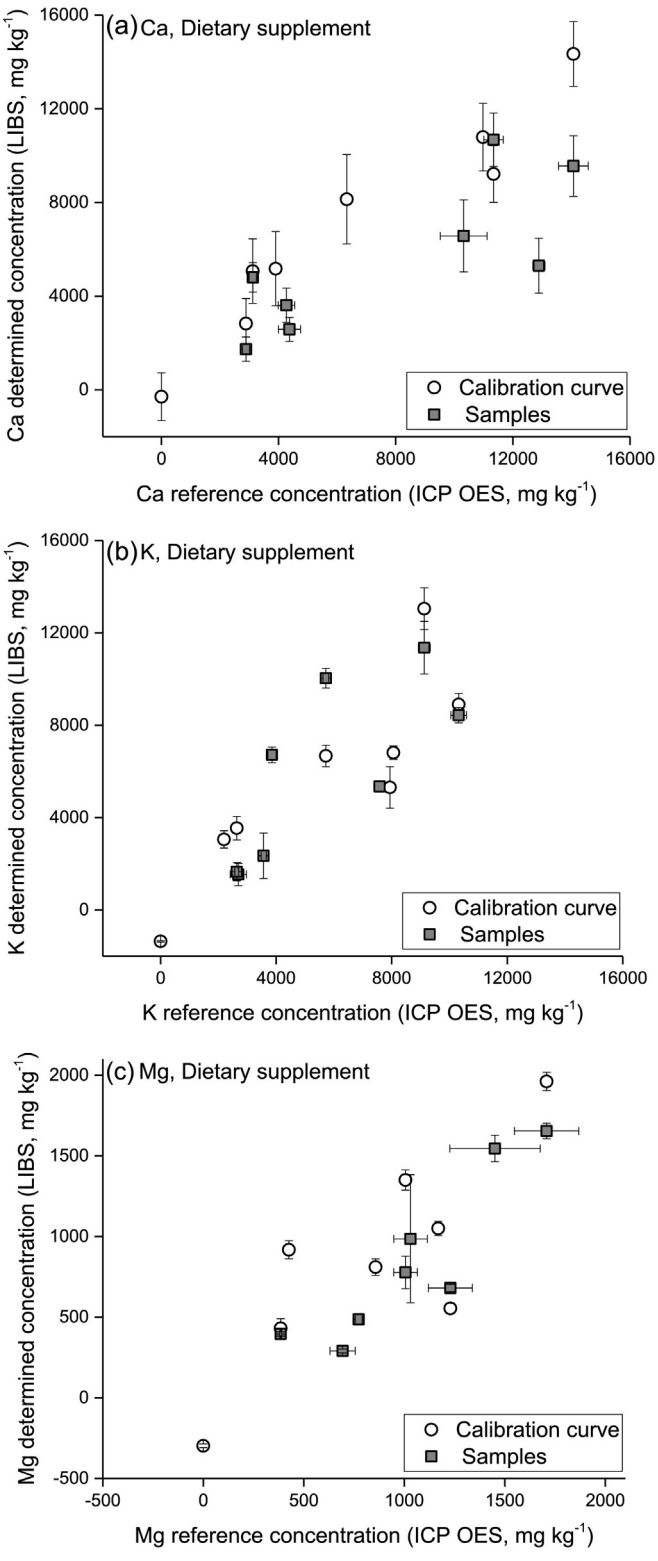

Fig. 4. Reference (ICP OES) and predicted (LIBS) concentrations (mg kg^{-1}) for solid dietary supplement analysis for Ca (a), K (b) and Mg (c) determination.

concentrations) and predicted (LIBS) values were compared was 0.6394. The SEC values ranged from 357 (for Mg) to 2137 mg kg^{-1} (for Ca).

Good concordance was observed for Ca, with the SE of prediction (SEP) equal to 3782 mg kg^{-1}. Accuracy varied from 57 to 176% for K and 42 to 107% for Mg. The SEP values were 2510 and 295 mg kg^{1} for K and Mg, respectively and were calculated according to Eq. (4):

$$SEP = \sqrt{\frac{(y_i - \hat{y}_i)^2}{n-1}} \quad (4)$$

where n is the number of samples and y_i and $\hat{y}_i$ are the reference (ICP OES) and the predicted analyte concentration for data set of prediction, respectively.

Fig. 4 shows the reference and predicted concentrations for solid dietary supplement samples (squares) and calibration (circles) curve.

The analytical frequency of the proposed method is around 60 samples per hour, without chemical toxic waste generated by using solvents and/or acids. The limits of quantification for the proposed LIBS method was calculated considering microcrystalline cellulose as blank and the values ranged from 49 (Mg) to 1955 (Ca) mg kg^{-1}.

4. Conclusions

The proposed method is suitable, fast and can be implemented for the direct determination of Ca, K, and Mg in solid food samples. In the case of Ca, limitations from matrix interference were minimized after dilution of the material using cellulose, case of the supplement samples. It was also concluded that the normalization process of the raw data plays an important role in the quality of the results.

Acknowledgement

This study was supported by the São Paulo Research Foundation (FAPESP), 2014/11415-0 PhD grant to ASA and 2016/00779-6, the National Council for Scientific and Technological Development (CNPq, 401074/2014-5, 305637/2015-0 and 445729/2014-7) and Pró-reitoria de Pesquisa (PROPe-UNESP, Grant 0268/001/14). The authors are grateful to Analítica and Thermo Scientific for the loan of instruments.

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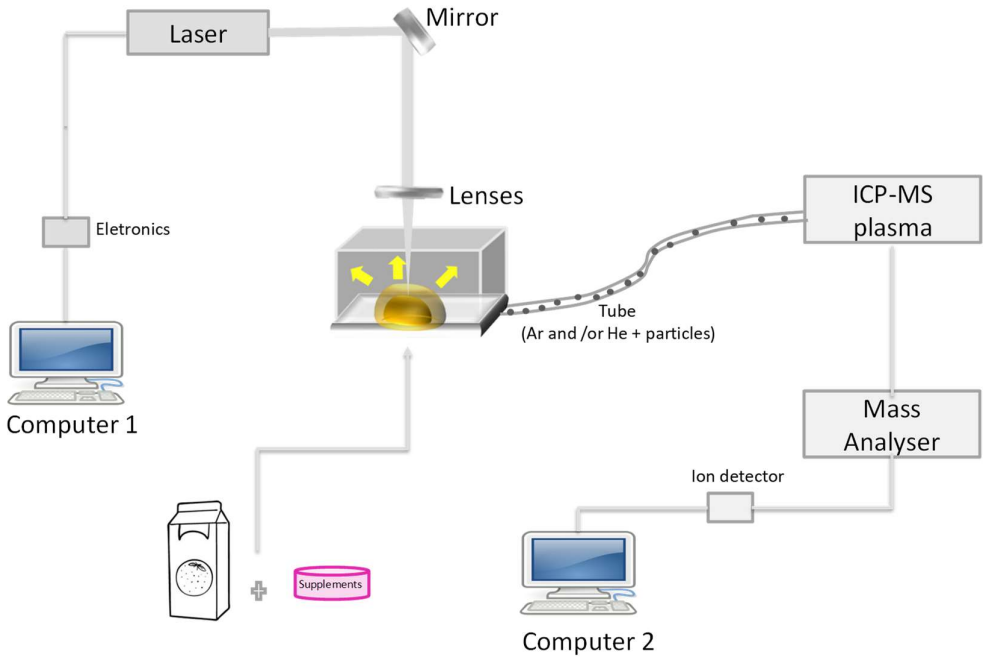
4.2 **"Nutrient and contaminant quantification in solid and liquid food samples using laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS): discussion of calibration strategies"**

Food Analytical Methods 10 (2017) 1515-1522.

Graphical Abstract



Food Anal. Methods (2017) 10:1515–1522
DOI 10.1007/s12161-016-0703-3

Nutrient and Contaminant Quantification in Solid and Liquid Food Samples Using Laser-Ablation Inductively Coupled Plasma-Mass Spectrometry (LA-ICP-MS): Discussion of Calibration Strategies

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Received: 13 October 2016 / Accepted: 17 October 2016 / Published online: 9 November 2016

Abstract The quality and safety of food samples always require strict control. From an analytical perspective, many techniques can be used for this type of monitoring, one of which involves the application of laser pulses in laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). LA-ICP-MS has great advantages such as simplicity of operation, versatility, and high analytical capability for multi-element determinations. One of the difficulties and challenges associated with this technique involves quantitative analyses because of problems in the process of ablation, plasma formation and/or matrix effects. In this case, calibration strategies combining internal standards, standard addition methods, and chemometric tools can help improve the results. Food samples are among the most variable samples in terms of their matrix and composition, which can further complicate analysis by laser ablation and require the development of ablation strategies. This study describes the use of calibration strategies and the determination of Ca, Cd, Cr, Cu, Fe, Mg, Pb and Zn in food samples, including liquid (i.e., orange juice) and solid (i.e., dietary supplements) samples. The calibration curve for the solid samples was constructed using microcrystalline cellulose and a proportional mixture of solid samples chosen according to their higher and lower concentrations of metals. The liquid samples were immobilized in a polymer film with the help of polyvinyl alcohol (PVA). Using these calibration strategies, it was possible to obtain accuracy values between 60 and 120 % for almost all samples, except for Ca, Mg, and Zn. These exceptions could be a reflection of unresolved matrix interferences. Carbon was used as the internal standard but did not show promising results.

Keywords LA-ICP-MS · Food samples · Calibration strategies

Electronic supplementary material The online version of this article (doi:10.1007/s12161-016-0703-3) contains supplementary material, which is available to authorized users.

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Introduction

Most people who seek a balanced diet can usually obtain nutrients from a healthy and adequate daily diet. Fruit juices are highly desirable, mainly due to their great taste and flavor, easy consumption, and exceptional nutritional qualities.

A diet rich in fruit juices is a good source of large amounts of essential and physiologically important nutrients, including vitamins, proteins, carbohydrates, and several elements required by infants, children, and adults (e.g., Al, Ca, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, P, and Zn) (Szymczycha-Madeja et al. 2014). However, even with a normal and healthy diet, it is necessary to use dietary supplements to compensate for deficiencies in macroelements and trace elements. The use of supplements may be required as a result of a highly selective diet in the case of pregnant women or in special cases such as children with a poor and restrictive diet (Wolle et al. 2014).

Several analytical techniques have been used for the quality assessment of these products and to monitor the content of potentially toxic elements. Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) is a widely applied technique that combines the direct sampling of solid materials by laser radiation and the high sensitivity and multielement

capability of ICP-MS (Darke and Tyson 1994). Laser ablation (LA) is a technique that avoids contamination or loss of analytes during preparation procedures because it offers direct analysis of solid samples with minimal sample preparation. Analysis by LA-ICP-MS requires a smaller amount of sample, provides fast sample exchange and throughput, and determines the spatial distributions of the elemental composition with low limits of detection (Russo et al. 2002). Moreover, the introduction mode of a sample without the typical solution components (dry plasma) into the ICP improves atomization and reduces spectral interferences and some matrix effects (Miliszkiewicz et al. 2015).

There are numerous advantages of LA-ICP-MS for direct solid sample analysis as mentioned. However, in general, the precision and accuracy of LA-ICP-MS are worse than those of typical ICP-MS analysis. To overcome crucial drawbacks related to the interactions between the laser beam and sample matrix, several normalization strategies have been developed over the years to address the different challenges and to allow sufficiently sensitive, reproducible, and accurate analysis (Sarkar et al. 2014; Castro and Pereira-filho 2016).

Among these strategies, the use of matrix-matched standards with reference materials is often recommended (Ohata et al. 2002). However, these procedures do not often cover all types of matrices or all ranges of analyte concentrations in the sample. To ensure sample and standard matching, the main sample components are usually used as a baseline for the standard preparation. Using this approach to adequately adjust the concentration ranges of the analytes in the prepared standard mixture for the elemental standards, the samples must be previously analyzed (Miliszkiewicz et al. 2015).

In the present study, a calibration method is proposed using LA-ICP-MS for the quantitative determination of nutrients and harmful elements in dietary supplement and fruit juice samples. The use of carbon as an internal standard to correct for the variability and signal fluctuations that occur during sampling and LA-ICP-MS analysis was investigated. In addition, the preparation of matrix-matched standards by mixing samples was assessed for dietary supplement calibration. A new strategy for the calibration and analysis of orange juice using a liquid-to-solid matrix conversion was also investigated.

Experimental

Reagents and Standards

All reagents were of analytical grade or higher purity. Deionized water (18.2 ΩM cm^{-1} resistivity), produced by a Milli-Q® Plus Total Water System (Millipore Corp., Bedford, MA, USA), was used to prepare all solutions. Multielement standard stock solutions, used for the calibration curve, were prepared from solutions of 10,000 mg L^{-1} Ca and Mg (SpecSol, Jacareí, SP, Brazil) and 1000 mg L^{-1} Cd (Qhemis, Jundiaí, SP, Brazil), Cr, Cu, Fe, Pb, and Zn (SpecSol).

Instrumentation and Reference Concentration Values

Both inductively coupled plasma-optical emission spectrometry (ICP OES, model iCAP 6000, Thermo Fisher Scientific, Madison, WI, USA) and ICP quadrupole mass spectrometry (ICP-MS, model iCAP Qc ICP-MS, Thermo Fisher Scientific, Madison, WI, USA) were used to obtain reference concentrations of Ca, Cd, Cr, Cu, Fe, Mg, Pb, and Zn in the liquid and solid samples. The ICP OES instrument allows sequential analytical signal collection using axial and radial viewings. The studied emission lines and operating equipment parameters are shown in Table S1 (see Supplementary Material).

Regarding ICP-MS, the instrument was equipped with a collision/reaction cell (QCell), and the monitored isotopes and technical operating parameters are shown in Table S2 (see Supplementary Material). In these determinations, argon (99.996 %, White Martins-Praxair, Sertãozinho, SP, Brazil) and helium (99.996 %, White Martins-Praxair, Sertãozinho, SP, Brazil) were used.

Before the ICP OES and ICP-MS determinations, the samples were subjected to microwave oven digestion in a Speedwave Four system (Berghof, Eningen, Germany). This instrument was equipped with 12 high-pressure TFM® vessels (DAP100), and the digestion mixture was composed of HNO_3 and H_2O_2 (30 % *w*/*w*) (Synth, Diadema, SP, Brazil). The concentrated HNO_3 was previously purified using a Distillacid™ BSB-939-IR sub-boiling distillation system (Berghof, Eningen, Germany).

The solid samples were accurately weighed using an analytical balance (model AY 220, max. of 220 g, 0.1 mg resolution, Shimadzu, Kyoto, Japan) and digested in a microwave oven, as previously mentioned. Two hundred and fifty milligrams of sample and 6 mL of HNO_3 (2 mol L^{-1}) were used. The final volume was adjusted to 50 mL with deionized water.

The liquid samples were also digested in a microwave oven. Three milliliters of sample, 5 mL of HNO_3 (2 mol L^{-1}), and 3 mL of H_2O_2 (30 % *w*/*w*) were used. The final volume was adjusted to 20 mL with deionized water, and the microwave heating program used for both samples (solid and liquid) is shown in Table S3 (see Supplementary Material).

Sample Description

Eight solid dietary supplement samples were purchased at a local market in São Carlos (São Paulo, Brazil). These samples were intended for consumption by children. In order to evaluate some figures of merit (precision and accuracy) of the

analytical techniques used, a reference material, NIST 1548a (typical diet), was employed.

The selected liquid samples included five ready-to-drink orange juice samples, which were also purchased at a local market in Sao Carlos.

Direct Solid Sample Analysis and Laser Ablation System

In the solid sample analyses by LA-ICP-MS, microcrystalline cellulose (P.A., Synth) was used to prepare standards after mixing with solid samples. In the case of liquid samples, a 10 % *w*/*v* solution of an aqueous polymer, polyvinyl alcohol (PVA) (99 % hydrolyzed, viscosity of 28–32 cP in a 4 % solution at 20 °C, Mw 85,000–124,000, Matheson Coleman & Bell), was employed to immobilize the liquid as a plain solid film.

A laser ablation system (model LSX-213 G2+, Teledyne CETAC Technologies, Omaha, NE, USA) coupled to a quadrupole-based ICP-MS instrument in standard mode was used to generate the solid particles. The laser system consisted of a Q-switched Nd:YAG laser (wavelength of 213 nm) with an energy of 4 mJ *per* pulse, a frequency from 1 to 20 Hz, and a spot size from 4 to 200 μm. This system was operated with three types of flow: He1 (helium flow responsible for transporting the ablated sample particles), He2 (helium flow responsible for cleaning the ablation cell and maintaining an inert atmosphere), and Ar (argon flow also responsible for transporting the sample). The operational conditions are shown in Table 1.

The computer program Matlab (MathWorks, Natick, Massachusetts, USA), version 2010, was used for organization and normalization of the dataset.

Calibration Strategy for Solid and Liquid Samples by LA-ICP-MS

Solid Samples

In the solid sample analysis, 500 mg was weighted and pressed into a pellet with a pressure of 10 t. Microcrystalline cellulose (P.A., Synth) was used as the blank and to prepare a calibration curve by mixing different samples. The calibration curve was prepared from a proportional mixture of four samples chosen according to higher and lower concentrations of metals.

Liquid Samples

For liquid sample analysis, multielement standard solutions were prepared and used for the calibration curve. Two hundred milligrams of the calibration curve or liquid samples was mixed with 800 mg of the 10 % *w*/*v* PVA solution. After homogenization, the mixtures were transferred to an aluminum holder over a glass support and dried in an oven at 50 °C for 2 h. After drying, a solid plain film was obtained for each point of the curve and for the five samples. The blank was a solution of nitric acid 1 % *v*/*v* also immobilized. All process mentioned previously are shown in Fig. 1.

LA-ICP-MS Analysis and Data Treatment

The laser ablation parameters were optimized to obtain signals with high sensitivity. This optimization was performed using both solid and liquid samples, and the selected parameters are listed in Table 1 (the variables studied are marked with an a).

Table 1 Laser ablation operational conditions for solid and liquid samples analysis

Instrumental parameter	Operational conditions	
	Solid samples	Liquid samples
Power percentage (%)[a]	40	60
Scan line (mm)	3	3
Spot size (μm)[a]	110	200
Repetition frequency (Hz)[a]	7	10
Scan rate (μm s^{-1})[a]	40	40
He1 flow (mL min^{-1})[a]	350	350
He2 flow (mL min^{-1})	200	200
Ar flow (mL min^{-1})[a]	250	250
Laser energy (mJ)	0.91	4.70
Fluence (J cm^{-2})	11.53	14.95
Irradiance (GW cm^{-2})	2.3	2.99
Mass/charge ratios monitored	^{12}C, ^{13}C, ^{25}Mg, ^{42}Ca, ^{53}Cr, ^{58}Fe, ^{65}Cu, ^{64}Zn, ^{111}Cd, ^{207}Pb	^{12}C, ^{13}C, ^{25}Mg, ^{42}Ca, ^{53}Cr, ^{58}Fe, ^{65}Cu, ^{64}Zn, ^{114}Cd, ^{207}Pb

[a] Variables studied using a fractional factorial design (2^{6-2})

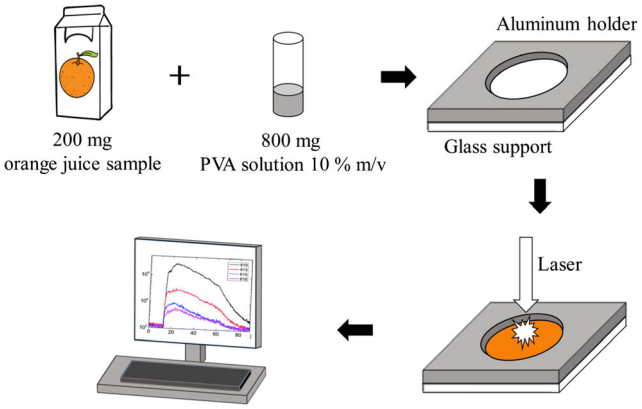

Fig. 1 Pictorial description of the procedure used to immobilize the liquid samples, blank, and calibration curve

These parameters were studied using a fractional factorial design of 2^{6-2}, and 16 experiments were performed.

In all samples, three lines 3 mm in length were ablated in line-scan mode, and for each measurement, a shutter delay of 10 s was established (gas blank). The signal acquisition time was 86 s. The signals obtained from each scan line were averaged, the signal area and height were calculated, and ^{12}C and ^{13}C were used as possible internal standards (IS).

Results and Discussion

Reference Values

Eight and five samples of dietary supplements and orange juice, respectively, were digested in triplicate (n = 3) and analyzed by ICP OES and ICP-MS. The major constituents (Ca, Cu, Fe, Mg, and Zn) were determined by ICP OES using both axial and radial views. The concentrations (mg kg^{-1}) of each wavelength (axial and radial view) are reported in Tables S4 (solid samples) and S5 (liquid samples) (see Supplementary Material). For the dietary supplements, the emission lines were selected based on the best recovery results from the NIST 1548a certified reference material. For the orange juice samples, the best emission lines were selected by comparing the results obtained in the radial and axial views, and the emission lines with the best concordance were selected. The average of the axial and radial view results for the selected lines was used to calculate the accuracy. These averages (reference values) are shown in Tables 2 and 3.

ICP-MS was used to determine the concentrations of Cd, Cr, and Pb. For Cd and Pb, only the most abundant isotopes or those that presented the best recovery from NIST 1548a (dietary supplements) were considered. For Cr, the isotope with the lowest possibility of interference according to the sample components was considered. All ICP-MS measurements were carried out in standard mode (no interference correction) and kinetic energy discrimination (KED) mode, which distinguish the interfering ions and by-product species (Thomas 2013) using a non-reactive gas, in this case, helium. The results presented (reference values) were only obtained in the KED mode and are reported in Tables 2 and 3.

Table S6 (see Supplementary Material) shows the selected emission lines, selected isotopes, coefficients of determination (R^2), and limits of quantification (LOQs) experimentally obtained by ICP OES and ICP-MS. To calculate the LOQ using ICP OES, the concept of background equivalent concentration (BEC), defined as the concentration of the analyte that produces a signal equivalent to the emission intensity of the background at the spectral line measured, was used (Schiavo et al. 2009). The LOQs for ICP-MS were calculated as 10 s/b, where s is the standard deviation (SD) of ten blanks and b is the slope of the calibration curve.

LA-ICP-MS Calibration for Solid and Liquid Food Samples

The ablated portions of the samples should represent the original sample, but factors such as homogeneity, laser wavelength, pulse duration, and fluency of the laser beam can cause fractionation (Günther and Hattendorf 2005). This fractionation affects all the calibration processes; however, two approaches can be used to overcome this problem: internal standardization and matrix matching (Lei et al. 2011). As mentioned previously, two strategies of calibration were used: one for the solid samples and one for the liquid samples. For the solid samples, a mixture of samples was used as the calibration curve, and for the liquid samples, a mixture of the samples and a PVA solution was used to obtain a solid film (Lin et al. 2016). All these strategies were employed in order to obtain a matched matrix and to avoid the drawbacks described above.

Figure 2 shows some examples of the calibration curves obtained for isotope ^{58}Fe in the solid samples (a) and ^{53}Cr in the liquid samples (b). As expected, the signals obtained for the solid calibration curve were noisier.

The area of each signal was calculated, and good linearity was obtained for the majority of the studied isotopes. The coefficient of determination (R^2) and LOQ are shown in Table S7 (see Supplementary Material). The LOQ values for LA-ICP-MS were calculated as 10 s/b, where s is the standard deviation (SD) of ten scan lines ablated in the blank (according to the type of sample) and b is the slope of the calibration curve. In this study, ^{12}C and ^{13}C were tested as an IS. The samples tested were carbon-rich, and the use of carbon as IS is an alternative (Nunes et al. 2016), although the results presented in the literature are inconclusive (Frick and Günther 2012).

Table 2 Determined and reference values, accuracies, and RSDs for solid samples ($n = 3$)

		Isotope							
		^{25}Mg	^{42}Ca	^{53}Cr	^{58}Fe	^{65}Cu	^{64}Zn	^{111}Cd	^{207}Pb
S1	Determined value (mg kg^{-1})	1122 ± 259	13,593 ± 3298	<LOQ	132 ± 24	4.4 ± 2.7	96 ± 35	<LOQ	<LOQ
	Reference value (mg kg^{-1})	773 ± 17	12,889 ± 206	0.2 ± 0.02	189 ± 12	10.1 ± 0.1	68 ± 1	0.013 ± 0.001	0.026 ± 0.004
	Accuracy (%)	145	105	–	70	44	142	–	–
	RSD (%)	25	25	–	5	30	29	–	–
S2	Determined value (mg kg^{-1})	1240 ± 206	13,646 ± 3262	<LOQ	155 ± 38	5.4 ± 3.4	94 ± 38	<LOQ	<LOQ
	Reference value (mg kg^{-1})	1229 ± 109	14,067 ± 506	0.61 ± 0.08	248 ± 19	13.2 ± 0.4	78 ± 4	0.023 ± 0.002	0.036 ± 0.001
	Accuracy (%)	101	97	–	63	41	121	–	–
	RSD (%)	19	25	–	14	42	33	–	–
S3	Determined value (mg kg^{-1})	463 ± 42	5308 ± 794	<LOQ	25 ± 19	2.2 ± 2.1	147 ± 45	<LOQ	<LOQ
	Reference value (mg kg^{-1})	693 ± 63	4374 ± 377	0.17 ± 0.03	250 ± 6	6.1 ± 0.2	172 ± 13	0.016 ± 0.005	0.017 ± 0.003
	Accuracy (%)	67	121	–	10	36	85	–	–
	RSD (%)	15	18	–	11	48	25	–	–
S4	Determined value (mg kg^{-1})	867 ± 164	3536 ± 1271	<LOQ	156 ± 27	4.6 ± 2.2	87 ± 43	<LOQ	<LOQ
	Reference value (mg kg^{-1})	1006 ± 59	2887 ± 119	0.9 ± 0.3	314 ± 9	10.2 ± 0.5	174 ± 13	0.027 ± 0.002	0.043 ± 0.003
	Accuracy (%)	86	122	–	50	46	50	–	–
	RSD (%)	22	39	–	6	8	43	–	–
S5	Determined value (mg kg^{-1})	1316 ± 99	9120 ± 1310	<LOQ	80 ± 42	4.1 ± 5.3	106 ± 80	<LOQ	<LOQ
	Reference value (mg kg^{-1})	1451 ± 225	10,319 ± 799	0.3 ± 0.07	155 ± 14	2.0 ± 0.3	98 ± 10	0.012 ± 0.0002	0.024 ± 0.001
	Accuracy (%)	91	88	–	52	205	108	–	–
	RSD (%)	10	16	–	39	158	73	–	–
S6	Determined value (mg kg^{-1})	443 ± 5	4317 ± 347	<LOQ	103 ± 58	2.6 ± 2.1	39 ± 5	<LOQ	<LOQ
	Reference value (mg kg^{-1})	1708 ± 160	11,340 ± 332	0.7 ± 0.01	126 ± 5	4.0 ± 0.6	101 ± 3	0.02 ± 0.001	0.027 ± 0.002
	Accuracy (%)	26	38	–	82	72	38	–	–
	RSD (%)	7	12	–	47	9	16	–	–
S7	Determined value (mg kg^{-1})	132 ± 90	2291 ± 1429	<LOQ	129 ± 106	2.2 ± 2.1	90 ± 35	<LOQ	<LOQ
	Reference value (mg kg^{-1})	385 ± 5	3121 ± 125	0.28 ± 0.007	155 ± 8	5.0 ± 0.2	76 ± 5	0.02 ± 0.01	0.019 ± 0.002
	Accuracy (%)	34	73	–	83	45	119	–	–
	RSD (%)	72	65	–	79	14	31	–	–
S8	Determined value (mg kg^{-1})	282 ± 99	2574 ± 737	<LOQ	155 ± 50	3.4 ± 2.5	68 ± 40	<LOQ	<LOQ
	Reference value (mg kg^{-1})	1031 ± 83	4267 ± 281	0.77 ± 0.05	251 ± 20	7.3 ± 0.4	155 ± 14	0.03 ± 0.002	0.054 ± 0.009
	Accuracy (%)	27	60	–	62	47	44	–	–
	RSD (%)	41	34	–	23	33	52	–	–
NIST 1548a	Determined value (mg kg^{-1})	233 ± 42	1123 ± 126	<LOQ	34 ± 21	2.4 ± 2.1	26 ± 15	<LOQ	<LOQ
	Reference value (mg kg^{-1})	595 ± 15	1871 ± 36	0.2 ± 0.02	25 ± 1	2.3 ± 0.07	25 ± 4	0.039 ± 0.007	0.1 ± 0.05
	Certified value (mg kg^{-1})	580 ± 27	1967 ± 113	–	35.3 ± 3.77	2.32 ± 0.16	24.6 ± 1.79	0.035 ± 0.0015	0.044 ± 0.009
	Accuracy (%)	39	60	–	137	109	106	–	–
	Recovery (%)	40	57	–	97	105	107	–	–
	RSD (%)	27	24	–	17	15	29	–	–

Table 3 Determined and reference values, accuracies, and RSDs for liquid samples ($n = 3$)

		Isotope							
		^{25}Mg	^{42}Ca	^{53}Cr	^{58}Fe	^{65}Cu	^{64}Zn	^{114}Cd	^{207}Pb
J1	Determined value (mg kg^{-1})	86 ± 14	90 ± 18	0.19 ± 0.11	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Reference value (mg kg^{-1})	137 ± 9	133 ± 8	0.23 ± 0.01	1.3 ± 0.1	2.4 ± 0.05	0.4 ± 0.03	<LOQ	<LOQ
	Accuracy (%)	63	67	84	–	–	–	–	–
	RSD (%)	13	7	2	–	–	–	–	–
J2	Determined value (mg kg^{-1})	76 ± 13	149 ± 19	0.39 ± 0.12	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Reference value (mg kg^{-1})	163 ± 9	417 ± 13	0.44 ± 0.03	1.5 ± 0.2	4.2 ± 0.1	0.4 ± 0.01	<LOQ	<LOQ
	Accuracy (%)	46	36	90	–	–	–	–	–
	RSD (%)	10	6	5	–	–	–	–	–
J3	Determined value (mg kg^{-1})	48 ± 1.5	141 ± 24	0.21 ± 0.12	<LOQ	<LOQ	7.1 ± 2.4	<LOQ	<LOQ
	Reference value (mg kg^{-1})	56 ± 3	170 ± 7	0.26 ± 0.003	1.3 ± 0.07	3.4 ± 0.03	5.6 ± 0.2	<LOQ	<LOQ
	Accuracy (%)	113	82	80	–	–	126	–	–
	RSD (%)	12	7	12	–	–	12	–	–
J4	Determined value (mg kg^{-1})	<LOQ	676 ± 48	0.38 ± 0.11	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Reference value (mg kg^{-1})	15 ± 0.4	746 ± 8	0.78 ± 0.04	0.3 ± 0.03	2.2 ± 0.04	0.1 ± 0.05	<LOQ	<LOQ
	Accuracy (%)	–	91	50	–	–	–	–	–
	RSD (%)	–	10	11	–	–	–	–	–
J5	Determined value (mg kg^{-1})	63 ± 18	49 ± 18	0.21 ± 0.12	<LOQ	<LOQ	7.6 ± 3.3	<LOQ	<LOQ
	Reference value (mg kg^{-1})	58 ± 2	37 ± 2	0.16 ± 0.002	0.5 ± 0.04	0.6 ± 0.02	0.2 ± 0.03	<LOQ	<LOQ
	Accuracy (%)	108	133	130	–	–	4115	–	–
	RSD (%)	14	6	12	–	–	24	–	–

An effective IS should present a behavior similar to the analyte during the process of ablation and in the ICP, i.e., the IS and analyte, must have similar characteristics, such as the atomic mass and/or first ionization potential (Austin et al. 2011; Frick and Günther 2012). The use of carbon as an IS presented no great advantage since a significant increase in the LOQs was observed, and the R^2 values were lower than 0.99 (Table S7). These results can be attributed to following: the first ionization potential of carbon is 11.26 eV, greatly different from all the analytes (6.11–9.39 eV), and the atomic mass of carbon is lower than the studied analytes.

Furthermore, the high intensity signals of ^{12}C and ^{13}C may have influenced the results. Austin et al. (Austin et al. 2011) proposed that the ^{13}C signal should not exceed 6 % of the total gross signal.

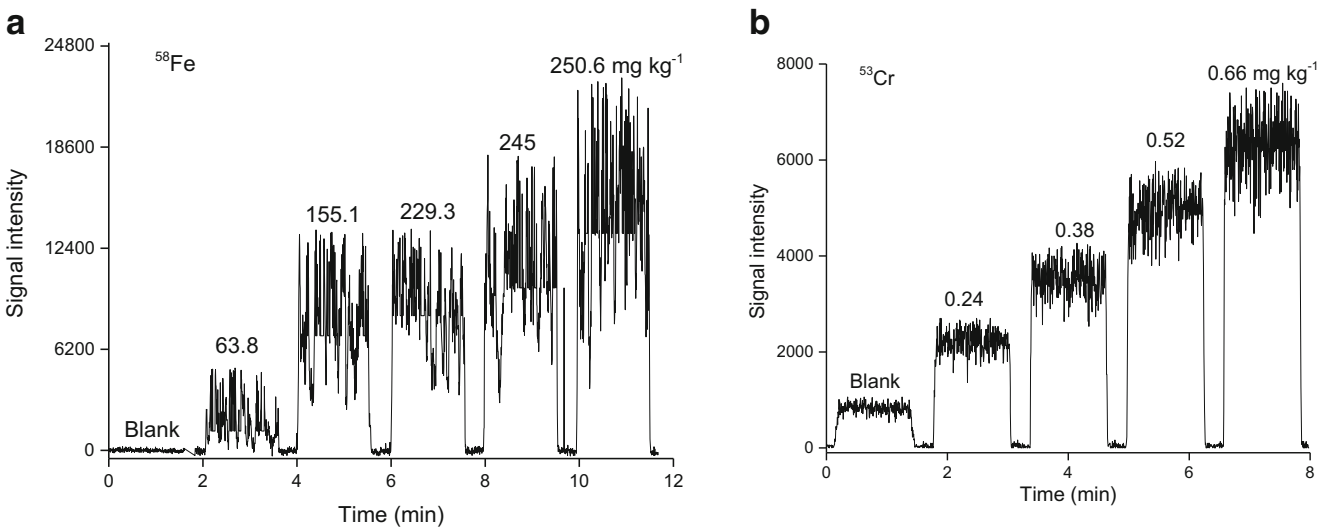

Fig. 2 Transient signals for ^{58}Fe and ^{53}Cr after laser ablation

Analysis of Solid and Liquid Food Samples by LA-ICP-MS

The concentrations of Ca, Cd, Cr, Cu, Fe, Mg, Pb, and Zn were determined by LA-ICP-MS and compared with the reference values obtained by ICP OES (Ca, Cu, Mg, Fe and Zn) and ICP-MS (Cd, Cr and Pb) in order to assess the feasibility of the proposed calibration strategy for the solid and liquid samples. Furthermore, the viability of the calibration strategy for the dietary supplements was also tested by analyzing a pellet of NIST 1548a; in this case, the accuracy (with the reference values) and recovery (with the certified value) were calculated.

Table 2 shows the data obtained for the solid samples: concentrations (LA-ICP-MS), reference values (ICP OES and ICP-MS), relative standard deviations (RSDs), accuracy, and recovery (NIST 1548a).

The RSDs for the macronutrients (Ca and Mg) ranged from 7 to 72 %, and the accuracy varied from 26 to 145 %. For ^{25}Mg, four samples (S2, S3, S4 and S5) of the nine analyzed (including NIST 1548a) presented accuracies from 67 to 101 % and RSDs ranging from 10 to 22 %. For ^{42}Ca, only one sample had low accuracy (38 %), and the accuracy and recovery for the NIST sample were 60 and 57 %, respectively.

Low accuracy can be due to the interference that occurs in ICP-MS as polyatomic and isobaric interferences. The combination of $^{12}C_2H$ is a potential polyatomic interference with the isotope ^{25}Mg, and combinations of $^{40}ArH_2$ and $^{13}C^{14}N^{16}O$ are polyatomic interferences with the isotope ^{42}Ca. It was not possible to determine ^{53}Cr because all concentrations were below the LOQ; combinations with carbon ($^{40}Ar^{13}C$) may create interferences in the same way as for Ca and Mg. The abundance of carbon in all samples from the cellulose and/or salts used to manufacture dietary supplements has hindered the determination of these elements.

In addition to carbon, other elements such as Cl, Na, O, and S may be added to dietary supplements using salts (Food Supplements 2012). These elements can also be responsible for polyatomic interferences with ^{58}Fe ($^{23}Na^{35}Cl$), ^{64}Zn ($^{32}S_2$ or $^{32}S^{16}O_2$) and ^{65}Cu ($^{32}S^{16}O_2H$ or $^{40}Ar^{25}Mg$).

The accuracy for ^{58}Fe ranged from 10 to 97 %, and the RSDs ranged from 5 to 79 %. Two samples presented accuracies of approximately 50 %, and only one sample accuracy was below 50 %. The accuracy and recovery for NIST 1548a were 137 and 97 %, respectively.

The accuracy for ^{64}Zn ranged from 60 to 120 % in four samples (S1, S6, S8 and S4), and the accuracy and recovery for NIST 1548a were 109 and 105 %, respectively. The RSDs ranged from 16 to 73 %. Only sample 6 and NIST 1548a presented accuracies above 60 % for ^{65}Cu, and the corresponding RSDs ranged from 8 to 158 %.

High RSDs can be explained by the characteristics of the samples (some samples were visibly heterogeneous) or inefficient homogenization during the preparation of pellets. Furthermore, the lack of homogeneity of the samples could have affected the accuracy results since fractionation was possible during the ablation process.

It was not possible to determine Cd and Pb, even using the most abundant isotopes, and the concentrations determined by LA-ICP-MS were below the LOQ.

Table 3 shows the data obtained for the liquid samples (juice): concentrations (LA-ICP-MS), reference values (ICP OES and ICP-MS), relative standard deviations, and accuracy.

In general, the RSDs ranged from 2 to 24 %, and the accuracy varied from 36 to 4115 %. The concentrations were below the LOQ for ^{58}Fe, ^{65}Cu, ^{114}Cd, and ^{207}Pb; thus, it was not possible to determine the concentrations of these analytes in all samples.

For ^{25}Mg and ^{42}Ca, only J2 presented accuracies below 60 %. The accuracy was 46 and 36 % for Mg and Ca, respectively. In J4, the concentration of ^{25}Mg was below the LOQ, and the accuracy for ^{42}Ca was above 120 % in J5. As mentioned previously, Ca and Mg can be affected by polyatomic interferences such as $^{40}ArH_2$ and/or $^{13}C^{14}N^{16}O$ and $^{12}C_2H$, respectively. These interferences can compromise the determination of these elements in a highly carbonaceous matrix such as plants (oranges). The RSDs of Ca and Mg in all samples ranged from 6 to 14 %.

Only two samples (J3 and J5) had concentrations above the LOQ for ^{64}Zn. The accuracy for J3 was 126 % and greatly exceeded 120 % for J5. As mentioned previously, polyatomic interferences from S ($^{32}S_2$ and/or $^{32}S^{16}O_2$), an essential nutrient for all plants, are responsible for characteristics such as smell and taste (Kopriva et al. 2015) and can influence the determination of ^{64}Zn. The RSDs of Zn were 12 % in J3 and 24 % in J5. The low RSDs demonstrate that the calibration strategy improved the precision of the results.

Conclusions

The two proposed calibration strategies appeared to be good alternatives for most of the samples (dietary supplements and orange juices). The observed accuracies were between 60 and 120 % for almost all the samples, and the observed exceptions were mainly for Ca, Mg, and Zn. Polyatomic interferences such as $^{32}S_2$ and/or $^{32}S^{16}O_2$ for ^{64}Zn, $^{40}ArH_2$ and/or $^{13}C^{14}N^{16}O$ for Ca, and $^{12}C_2H$ for Mg were associated with low accuracy values. The use of carbon as an internal standard to address matrix interferences did not yield promising results. The higher concentration of carbon compared with the monitored analytes probably prevented the use of this internal standard, in addition to the differences in atomic mass and/or

first ionization potential. All results demonstrate that the proposed method can be easily applied in preliminary tests for food sample inspection.

Acknowledgments This study was supported by the Sao Paulo Research Foundation (FAPESP), 2015/14488-0, 2014/11415-0 PhD grant to ASA, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 401074/2014-5, 160152/2015-1 and 305637/2015-0). The authors are grateful to Analítica and Thermo Scientific for the instrument loan.

Compliance with Ethical Standards

Conflict of Interest Amanda dos Santos Augusto declares that she has no conflict of interest. Marco Aurélio Sperança declares that he has no conflict of interest. Daniel Fernandes de Andrade declares that he has no conflict of interest. Edenir Rodrigues Pereira-Filho declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human or animal subjects.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Supplementary material

Table S1. ICP OES instrumental conditions for wet digestion of samples analysis

ICP OES	
Characteristics	**Parameters**
Integration time for low and high emission lines (s)	5
Sample introduction flow rate (mL min^{-1})	2.1
Sample flow rate during analyses (mL min^{-1})	2.1
Pump stabilization time (s)	25
Radio frequency applied power (W)	1200
Auxiliary gas flow rate (L min^{-1})	0.25
Nebulization gas flow rate (L min^{-1})	0.83
Cooling gas flow rate (L min^{-1})	16
Emission lines for the analytes in axial and radial views (nm)	**Ca** (184.0**, 317.9**, 422.6*, 431.8*), **Cd** (214.4**, 226.5**, 228.8*), **Cr** (267.7**, 283.5**, 284.3**, 357.8*), **Cu** (217.8**, 221.8**, 224.7**, 324.7*, 327.3*), **Fe** (238.2**, 239.5**, 240.4**, 259.8**, 259.9**), **Mg** (279.5**, 280.2**, 285.2*, 382.9*), **Pb** (216.9*, 220.3**, 261.4*), **Zn** (202.5**, 206.2**, 213.8*, 481.0*)

*Atomic emission lines
**Ionic emission lines

Table S2. ICP-MS instrumental conditions for element determinations

ICP-MS	
Characteristics	**Parameters**
Cooling gas flow rate (L min^{-1})	14
Auxiliary gas flow rate (L min^{-1})	0.8
Nebulization gas flow rate (L min^{-1})	1.18
Radio frequency applied power (W)	1550
CCT1 He flow (mL min^{-1})	4.5
D1 lenses (V)	-194
D2 lenses (V)	-80
CCT focus lenses (V)	-0,9
Metering mode	STD and KED
Monitored isotopes	^{24}Mg, ^{25}Mg, ^{26}Mg, ^{42}Ca, ^{43}Ca, ^{44}Ca, ^{48}Ca, ^{50}Cr, ^{52}Cr, ^{53}Cr, ^{57}Fe, ^{58}Fe, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn, ^{70}Zn, ^{112}Cd, ^{114}Cd, ^{206}Pb, ^{207}Pb, ^{208}Pb

Table S3. Microwave oven parameters for solid and liquid sample preparation

	Solid Samples (Dietary Supplements)				Liquid Samples (Orange Juice)			
	Power (W)	Temperature (°C)	Ramp (min)	Time (min)	Power (W)	Temperature (°C)	Ramp (min)	Time (min)
1	1260	120	5	5	870	150	4	15
2	1260	160	5	5	1160	210	4	25
3	1260	230	5	10	-	-	-	-

Table S4. Reference values for the analytes determined in the solid samples (n=3)

		Reference values for solid samples (mg kg^{-1})								
		S1	S2	S3	S4	S5	S6	S7	S8	NIST 1548a
ICP OES (Axial view)	Ca 317.9 nm	12849±221	14309±583	4431±340	2924±96	11457±716	12232±59	2962±68	4060±240	1807±51
	Cu 324.7 nm	10.1±0.2	13.4±0.3	6.3±0.3	10.8±0.5	2.2±0.02	4.2±0.7	5.1±0.4	7.3±0.4	2.0±0.04
	Cu 327.3 nm	10.2±0.2	13.5±0.3	6.5±0.3	10.8±0.5	2.4±0.1	4.4±0.5	4.9±0.4	7.3±0.3	2.1±0.06
	Fe 238.2 nm	189±12	249±20	253±7	318±10	159±15	127±0.4	153±7	247±19	35±1
	Fe 239.5 nm	191±12	250±21	253±7	317±9	159±15	128±1	153±7	247±19	35±1
	Fe 259.9 nm	188±12	243±18	244±6	307±7	147±13	124±12	159±9	258±21	35±2
	Mg 279.5 nm	805±20	1432±66	779±64	1275±69	2043±174	2551±26	334±9	1079±86	576±18
	Mg 280.2 nm	741±17	1296±52	712±54	1142±51	1787±126	2226±7	359±6	1017±76	559±16
	Mg 285.2 nm	691±10	1122±18	574±43	919±8	1321±36	1527±17	395±4	1050±88	565±11
ICP OES (Radial view)	Ca 317.9 nm	12929±192	13826±429	4316±414	2850±143	9180±883	10449±604	3281±183	4473±323	1934±21
	Cu 324.7 nm	9.8±0.3	12.9±0.5	5.6±0.2	9.3±0.5	1.5±0.7	2.7±0.7	4.8±0.1	7.2±0.5	2.4±0.06
	Cu 327.3 nm	10.10±0.04	13.0±0.5	5.8±0.1	9.7±0.4	2.0±0.5	3.2±0.4	4.6±0.1	7.2±0.6	2.6±0.1
	Mg 279.5 nm	878±23	1292±223	790±103	937±125	1391±436	1578±405	404±2	1009±76	640±14
	Mg 280.2 nm	852±22	1258±211	793±80	998±97	1399±454	1577±437	404±2	989±75	630±13
	Mg 285.2 nm	669±8	974±82	510±34	765±5	767±128	787±69	413±7	1041±99	601±17
	Zn 202.5 nm	70±1	81±5	182±14	184±16	107±12	114±3	75±5	152±14	24±4
	Zn 213.8 nm	66±1	74±3	162±12	163±10	89±9	89±3	76±5	157±15	25±4

Table S5. Reference values for the analytes determined in the liquid samples (n=3)

		Reference values for liquid samples (mg kg^{-1})				
		J1	J2	J3	J4	J5
ICP OES (Axial view)	Ca 422.6 nm	136±8	439±15	192±7	755±14	40±2
	Cu 221.8 nm	2.10±0.07	3.7±0.2	3.2±0.04	2.1±0.02	0.6±0.02
	Fe 240.4 nm	1.1±0.05	1.3±0.06	1.3±0.01	0.3±0.004	0.5±0.02
	Mg 280.2 nm	124±9	152±11	49±1	13±1	60±2
	Mg 285.2 nm	143±10	175±15	52±1	15±0.5	61±2
	Zn 206.2 nm	0.4±0.02	0.4±0.02	5.4±0.1	0.2±0.04	0.2±0.02
	Zn 213.8 nm	0.4±0.03	0.5±0.02	5.7±0.1	0.2±0.05	0.2±0.03
ICP OES (Radial view)	Ca 422.6 nm	131±8	395±11	149±6	737±2	35±2
	Cu 221.8 nm	2.7±0.04	4.7±0.06	3.5±0.02	2.2±0.07	0.6±0.02
	Fe 240.4 nm	1.5±0.2	1.7±0.3	1.3±0.1	0.2±0.05	0.5±0.07
	Mg 280.2 nm	137±7	159±5	43±1	16±0.2	55±2
	Mg 285.2 nm	144±9	171±5	47±2	17±0.1	57±3
	Zn 206.2 nm	0.4±0.04	0.4±0.01	5.6±0.2	0.1±0.06	0.1±0.03
	Zn 213.8 nm	0.4±0.04	0.4±0.02	5.7±0.2	0.1±0.05	0.1±0.03

Table S6. Selected figures of merit for the proposed methods

	Solid Samples			Liquid Samples		
		LOQ (mg kg^{-1})	R^2		LOQ (mg kg^{-1})	R^2
Isotope (ICP-MS)	^{53}Cr	0.02	0.99	^{53}Cr	0.005	0.99
	^{111}Cd	0.0008	0.99	^{114}Cd	0.0002	0.99
	^{207}Pb	0.0005	0.99	^{207}Pb	0.00008	0.99
Wavelength (ICP OES)	Ca 317.9 nm*	0.08	0.99	Ca 422.6 nm*	1.0	0.99
	Ca 317.9 nm**	0.09	0.99	Ca 422.6 nm**	0.3	0.99
	Cu 324.7 nm*	0.0015	0.99	Cu 221.8 nm*	1.3	0.99
	Cu 324.7 nm**	0.0020	0.99	Cu 221.8 nm**	1.4	0.99
	Cu 327.3 nm*	0.0010	0.99	Fe 240.4 nm*	0.04	0.99
	Cu 327.3 nm**	0.0046	0.99	Fe 240.4 nm**	0.30	0.97
	Fe 238.2 nm*	0.11	0.99	Mg 280.2 nm*	0.09	0.99
	Fe 239.5 nm*	0.11	0.99	Mg 280.2 nm**	0.02	0.99
	Fe 259.9 nm*	0.12	0.99	Mg 285.2 nm*	0.07	0.99
	Mg 279.5 nm*	0.008	0.99	Mg 285.2 nm**	0.02	0.99
	Mg 279.5 nm**	0.004	0.99	Zn 206.2 nm*	0.05	0.99
	Mg 280.2 nm*	0.006	0.99	Zn 206.2 nm**	0.04	0.99
	Mg 280.2 nm**	0.004	0.99	Zn 213.8 nm*	0.05	0.99
	Mg 285.2 nm*	0.006	0.99	Zn 213.8 nm**	0.05	0.99
	Mg 285.2 nm**	0.003	0.99	-	-	-
	Zn 202.5 nm**	0.05	0.99	-	-	-
	Zn 213.8 nm**	0.04	0.99	-	-	-

*axial view **radial view

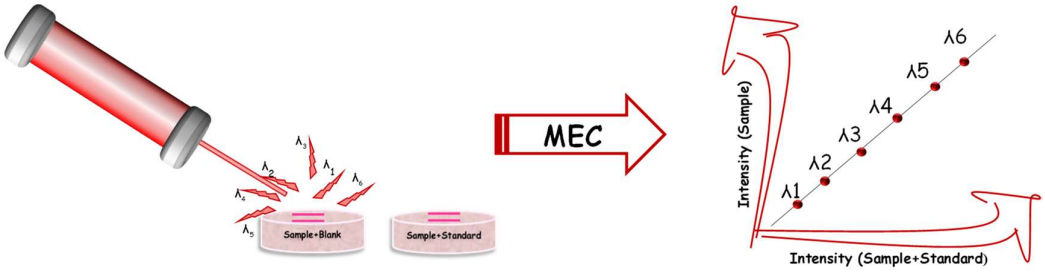
Table S7. Selected figures of merit for the proposed LA-ICP-MS method

Determined by LA-ICP-MS							
	Without ^{12}C and ^{13}C as IS			With ^{12}C as IS		With ^{13}C as IS	
	Isotope	LOQ (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	R^2	LOQ (mg kg^{-1})	R^2
Liquid Samples	^{25}Mg	25	0.99	28	0.98	28	0.98
	^{42}Ca	37	0.98	40	0.98	40	0.98
	^{53}Cr	0.2	0.99	29	0.10	32	0.10
	^{58}Fe	1.6	0.95	1.6	0.82	1.6	0.82
	^{65}Cu	8.1	0.98	0.0004	0.22	0.04	0.22
	^{64}Zn	4.4	0.98	4.4	0.72	4.5	0.72
	^{114}Cd	1.2	0.99	0.0003	0.40	0.02	0.40
	^{207}Pb	0.1	0.99	5.8	0.70	5.8	0.70
Solid Samples	^{25}Mg	16	0.98	3758	0.99	2999	0.99
	^{42}Ca	1308	0.98	28105	0.96	26799	0.98
	^{53}Cr	1.3	0.95	2.3	0.93	2.7	0.86
	^{58}Fe	19	0.97	26	0.94	26	0.96
	^{65}Cu	0.2	0.95	0.3	0.85	0.3	0.87
	^{64}Zn	3.6	0.95	4.5	0.98	5.2	0.95
	^{114}Cd	0.5	0.81	0.8	0.68	0.8	0.70
	^{207}Pb	0.07	0.99	0.08	0.99	0.08	0.99

4.3 "Combination of multi-energy calibration (MEC) and laser-induced breakdown spectroscopy (LIBS) for dietary supplements analysis and determination of Ca, Mg and K"

Journal of the Brazilian Chemical Society, DOI: http://dx.doi.org/10.21577/0103-5053.20180211

Graphical Abstract (GA) Figure:



GA Text:

Ablation process by LIBS on dietary supplement surface and multi-energy calibration method (MEC)

http://dx.doi.org/10.21577/0103-5053.20180211

J. Braz. Chem. Soc., Vol. 00, No. 00, 1-9, 2018
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Article

Combination of Multi-Energy Calibration (MEC) and Laser-Induced Breakdown Spectroscopy (LIBS) for Dietary Supplements Analysis and Determination of Ca, Mg and K

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This study describes the application of laser-induced breakdown spectroscopy (LIBS) for the direct determination of Ca, K and Mg in powdered dietary supplements. Multi-energy calibration (MEC) method was applied to obtain a calibration curve. With MEC, it was possible to observe spectral interferences and select adequate emission lines from LIBS. For Ca and Mg, five lines were selected and for K just two lines among four could be selected (compromising the results). The trueness for dietary supplements ranged from 81 to 103% for Ca and 74 to 106% for Mg. For K, just the samples S3 (95%) and S5 (109%) showed acceptable trueness values. In the case of Ca and K, besides the MEC, the normalization using C as internal standard also improved the figure of merit results. The MEC and normalization processes showed that possible matrix effect and spectral interferences could be avoided, and the results of trueness and precision were satisfactory.

Keywords: multi-energy calibration, direct solid analysis, dietary supplements, LIBS, macronutrients determination

Introduction

The most exploited aspect in analytical chemistry is related to quantitative analysis. These analyses are mainly based on modern instrumental techniques, which are able to record analytical signals intensities for a single analyte in a short time interval. The majority of analytical procedures employ aqueous samples and standards and a myriad of calibration strategies is routinely available in the literature. The most common and successfully employed one is external calibration, where several standard solutions with different concentrations of the analyte is used to propose a linear model using analyte standard concentrations in *x*-axis and signal intensities in *y*-axis. Afterwards, a linear model is calculated and used to obtain the analyte concentration of samples and some figures of merit, such as limits of detection (LOD) and quantification (LOQ).[1,2]

The limitation of external calibration is the fact that full analyte selectivity is required and the influence of sample matrix must be negligible.[1] In all cases reported in the literature, an intense sample preparation procedure is required. These procedures include wet digestion protocols that employ a simple sample dissolution (feasible in few cases),[3-7] digester block[8-12] or microwave oven assisted digestion.[3,5,13-15] Results from samples with a complex matrix can be strongly affected if the differences between sample and standard matrices are neglected. Beside the external calibration, alternatives such as internal standard (IS) method[16-19] and standard addition method[20-24] are often used in analytical chemistry.

All the traditional calibration methods previously mentioned present drawbacks for analysis of complex samples, being in some cases necessary the application of other strategies such as multivariate calibration[25-29] and matrix-matching procedures.[30-32]

Since 2011, nontraditional calibration methods, such as standard dilution analysis (SDA),[30-33] interference standard method (ISM)[34-36] and multi-energy calibration (MEC),[37] have been applied for quantitative analysis by inductively coupled plasma (ICP) optical emission spectrometry (OES), high-resolution continuum source (HR-CS) flame atomic absorption spectrometry (FAAS), ICP-mass spectrometry (MS) and microwave induced plasma (MIP) with OES.

According to Virgilio *et al.*,[37] MEC is a method where the signal intensity for a wavelength can be directly correlated to the concentration of the analyte and the

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excited-state of energy. In the procedure proposed by Virgilio *et al.*,[37] a simple and efficient matrix-matching procedure was proposed for several types of samples after wet digestion or dissolution (e.g., green tea, beer, red wine, apple juice, cola soft drink, vinegar, ethanol fuel and creek water) using ICP OES, HR-CS FAAS and MIP OES.

As widely reported in the scientific literature, a matrix-matching procedure in analysis is essential, mainly for complex samples, as food. These difficulties are even higher when direct solid sample analysis is performed, and several strategies are reported in the literature.[16,17,38]

Beyond the matrix complexity, most samples require an acid digestion process to be analyzed by conventional techniques. During sample preparation, errors can be introduced due to the several unitary operations, such as dilution and acid addition that compromise analytical frequency increasing the contamination possibilities and generating residues.[39]

Sample preparation processes can be avoided or minimized when analytical techniques that allow the direct solid sample analysis are applied, being laser-induced breakdown spectroscopy (LIBS) one alternative.[40-43] Besides the advantage mentioned, the LIBS analysis has high analytical frequency, requires reduced sample mass (typically less than 100 mg), and has multi-element capability when compared with FAAS.[42]

On the other hand, disadvantages related to calibration method are observed because the ablation process involves some μg of samples. Reference material with certified values concentration for masses in this range or lower are not commercially available.[38,44,45] Direct solid analysis also presents difficulties, such as the data reproducibility related to the ablation process, formation of the plasma, microheterogeneity and matrix effects, which in some cases can be minimized applying several types of normalizations or standardization on the raw data.[46]

In the present study, a simple and fast method for the direct analysis of powdered samples of dietary supplements by LIBS to determine Ca, Mg and K was applied and discussed. The dietary supplements, including those analyzed in this study, are commonly used in the sense of compensate possible deficiencies in macro- and trace elements. These products are prepared synthetically in laboratories with the addition, for example, of powdered milk, maltodextrin, sucrose, cellulose, vitamins (i.e., ascorbic acid) and minerals (i.e., calcium carbonate ($CaCO_3$), calcium phosphate, magnesium carbonate ($MgCO_3$), magnesium phosphate, potassium iodide (KI)). The spectra obtained from LIBS technique present several emission lines allowing to explore the MEC capability as a strategy to obtain the concentration values and circumvent problems related to matrix effects. Also, several types of normalization modes, including the use of IS naturally presented in the sample (mainly carbon), were tested to improve figures of merit.

Experimental

Reagents, sample description and reference values acquisition

The reagents used throughout the study were of analytical grade and higher purity. For the ICP OES analysis the water used was deionized using a Milli-Q® Plus Total Water System (18.2 MΩ cm resistivity; Millipore Corp., Bedford, MA, USA). All flasks (polypropylene (PP)) and glassware were previously decontaminated by soaking into a 10% v v^{-1} HNO_3 solution for 24 h and rinsed with deionized water afterwards. Multi-element standard solutions were prepared daily after successive dilutions of stock solutions: 10,000 mg L^{-1} Ca, and 1,000 mg L^{-1} K and Mg (Quemis, Jundiaí, SP, Brazil). These multi-element solutions were used to prepare the calibration curves to obtain reference concentration values in ICP OES determinations. Six commercial solid dietary supplements (S1-S6) were analyzed and further details about the intended use can be found elsewhere.[28]

For the ICP OES determinations, the solid samples were submitted to wet digestions with the assistance of a microwave equipment, employing analytical grade concentrated (14 mol L^{-1}) HNO_3 (Synth, Diadema, SP, Brazil) that was previously sub-boiled with a Distillacid™ BSB-939-IR sub-boiling system (Berghof, Eningen, Germany) and 30% m v^{-1} H_2O_2 (Synth) was used as auxiliary oxidant reagent. Speedwave Four microwave system (Berghof) used was equipped with eight high pressure with eight high-pressure TFM® vessels (DAK100). The acid digestion procedure was accomplished with 500 mg sample (pellets used for the LIBS analysis), 6 mL of HNO_3 (2 mol L^{-1}) and 3 mL of H_2O_2. The heating program is described in Table S1 (Supplementary Information (SI) section).

The measurements were performed by Thermo iCAP 7000 ICP OES system (Thermo Fisher Scientific, Madison, MT, USA) and an external calibration was applied to obtain the reference concentration values of Ca, K and Mg in the dietary supplements samples. All operational parameters from this system are shown in Table S2 (SI section).

LIBS instrumentation and solid sample preparation

The LIBS instrument used in the present study is a commercial benchtop system, model J200 (Applied

Spectra, Freemont, CA, USA). The system is equipped with a 1064-nm neodymium-doped yttrium aluminum garnet (Nd:YAG) laser with a pulse duration of 8 ns. The spectrometer is a 6-channel charged coupled device (CCD) with 12,288 pixels ranging from 186 to 1042 nm. The operational conditions of the LIBS instrument can be varied in the following ranges: (*i*) gate delay from 0 to 2 μs; (*ii*) laser pulse energy from 0 to 100 mJ; (*iii*) spot size from 50 to 250 μm; (*iv*) gate width is fixed in 1.05 ms; and (*v*) laser pulse repetition rate, adjustable from 1 to 10 Hz.

With the combination of these parameters the laser pulse irradiance (GW cm^{-2}) and fluence (mJ cm^{-2}) can range from 0.255 GW cm^{-2} and 2 mJ cm^{-2} (250 μm spot size and 1 mJ laser pulse energy) to 636.62 GW cm^{-2} and 5093 mJ cm^{-2} (50 μm spot size and 100 mJ laser pulse energy). The power ranges from 125 kW (1 mJ laser pulse energy) to 12.5 MW (100 mJ laser pulse energy).

Several different food matrices are currently analyzed in our research group using this instrument and the conditions are well stablished with several examples obtained in the last 3 years.[12,28,44] In this way, Table 1 shows an instrumental condition that permitted good reproducibility, no signal saturation for major constituents and high analytical frequency.

Table 1. Experimental conditions for the J200 LIBS measurements

Parameter	Value
Delay time / μs	0.5
Spot size / μm	50
Laser pulse energy / mJ	50
Fluence / (mJ cm^{-2})	2546
Irradiance / (GW cm^{-2})	318
Sample speed / (mm s^{-1})	1
Laser repetition rate / Hz	10
Number of scan lines	20
Distance between lines / mm	0.5
Approximate number of laser pulses *per* line	70
Total spectra recorded *per* pellet	around 1400

In order to perform the LIBS measurements and following MEC assessment, $CaCO_3$ (100.09 g mol^{-1}, Mallinckrodt, Staines-upon-Thames, UK), $MgCO_3$ (84.32 g mol^{-1}, ECIBRA, Curitiba, PR, Brazil), KI (166.00 g mol^{-1}, Synth), and microcrystalline cellulose ($C_6H_{10}O_5$, 324.3 g mol^{-1}, density: 0.26-0.34 g cm^{-3}; Synth) were used to prepare standards and pelletized.

In this study, cellulose was considered as blank and for the standards, a solid mixture with cellulose was previously prepared to obtain an intermediary stock solid mixture. The final Ca, K and Mg concentrations were 0.707, 0.595 and 0.182% m m^{-1}.

For MEC assessment, two solid mixtures (pellets) are needed: (*i*) pellet 1 (sample plus blank (microcrystalline cellulose)); and (*ii*) pellet 2 (sample plus stock mixture (microcrystalline cellulose and the salts of Ca, K and Mg)).

For all samples preparation, 250 mg of each one was mixed in a mortar with 250 mg of cellulose (pellet 1: sample + blank) or 250 mg of standard stock solid mixture (pellet 2: sample + standard), then pelletized using about 10 t $inch^{-1}$ of pressure with a pressing machine. All pellets were made in triplicate. In total, for the six samples, 72 pellets were prepared. Figure 1 shows all the procedure mentioned in this section.

Additional tests were performed changing the proportion of the analytes in the stock mixture. In this case, four mixtures were prepared with the following concentrations (in %): (*i*) Ca 0.66, K 0.68, Mg 0.18; (*ii*) Ca 0.35, K 0.60, Mg 0.18; (*iii*) Ca 0.72, K 0.26, Mg 0.18; and (*iv*) Ca 0.72, K 0.59, Mg 0.09. The goal of this test was to observe if the MEC approach can correct different analytes proportions in the standard mixture.

The data treatment was performed in Microsoft Excel® and MATLAB 2017b.[47]

Results and Discussion

As mentioned before, new calibration strategies such as MEC can be applied to avoid difficulties related to sample

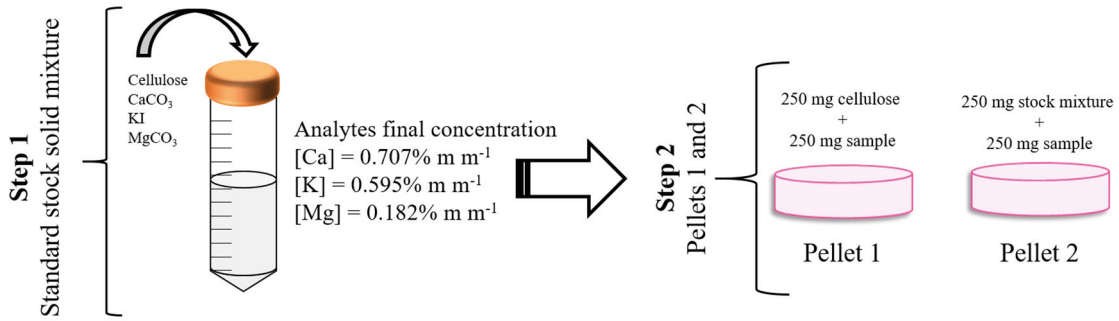

Figure 1. Sample preparation procedure for LIBS analysis using MEC.

matrix, since this strategy is based on a matrix-matching process. In this sense, MEC is a new alternative for solid analysis by LIBS. Figure 1 shows that the amount of sample added in both pellets (1 and 2) is the same. Direct solid analysis using LIBS is strongly affected by matrix interferences, mainly for food samples, which is a complex matrix due to their organic compounds such as lipids, carbohydrates and proteins.[48]

LIBS analysis can provide a lot of spectral information and chemometric tools are required to assess and improve the results. In this way, before MEC, twelve normalization modes were also applied to the raw spectral information of each sample and standards.

In this section, the twelve normalizations were codified as 1 (average), 2 (norm and average), 3 (area and average), 4 (individual spectrum maximum and average), 5 (sum), 6 (norm and sum), 7 (area and sum), 8 (individual spectrum maximum and sum), 9 (C I 193.09 nm (atomic line) as internal standard (IS) and average), 10 (C I 193.09 nm as IS and sum), 11 (C I 247.85 nm (atomic line) as IS and average) and 12 (C I 247.85 nm as IS and sum). It is important to mention that the normalization number 1 is only the average of all analytical signals. Our research group is investigating these standardization strategies since 2016 and detailed information can be assessed in the studies published by Castro and Pereira-Filho[46] and Sperança *et al.*[49]

After data normalizations, values of area and height were calculated for each analyte and the MEC was applied for selected emission lines. At the first attempt, eight emission lines for Ca and Mg, and four for K were evaluated, considering those that presented the highest relative intensity. Due to the capability of MEC to identify spectral interferences, few emission lines were removed to improve the statistical parameters (determination coefficient, R^2) of the calculated linear models. Figure 2 shows an example using sample S1. This figure shows the selected emission lines for each analyte: sample + cellulose (*y*-axis, pellet 1) and sample + standard (*x*-axis, pellet 2) and linear model for the signal height normalized by norms 9, 11 and 8 for Ca, K and Mg, respectively.

As can be noted in Figures 2a and 2b, five emission lines were selected for Ca. As expected, Ca signals in the pellet 2 (sample + standard) are greater than those observed for pellet 1 (sample + cellulose). From the eight emission lines tested, two presented spectral interferences (396.84 and 215.88 nm) and the line 534.94 nm presented low intensity signal. Four figures of merit were calculated for each sample and for each normalization mode: slope from the linear model, uncertainty with 95% of confidence level (n = 3), relative standard deviation (RSD, n = 3) and trueness calculated after comparison between the reference (ICP OES) and predicted concentrations. The slope was calculated according to the linear model stablished for each sample as depicted in Figures 2b (Ca), 2d (K) and 2f (Mg).

LIBS concentration values (C^{Sam}) for each analyte, sample and normalization were calculated by equation 1.

$$C^{Sam} = \frac{\text{slope} \times C^{Std}}{(1 - \text{slope})} \quad (1)$$

where C^{Std} is known and constant.

Equation 2 represents an example using the equation 1 to calculate the Ca concentration by LIBS for one replicate of sample 1. The signal used was the height and normalization 9 (C I 193.09 nm).

$$C^{Sam} = \frac{0.503 \times 0.707\% \text{ m m}^{-1}}{(1 - 0.503)} = 0.715\% \text{ m m}^{-1} \quad (2)$$

In order to evaluate the contribution of the errors related to the linear model calculated, an analysis of variance (ANOVA) was performed for each replicate. The uncertainty obtained was propagated and a 95% of confidence was applied to the final value. The values of uncertainty and RSD were calculated for three replicates, for each sample and normalization (see calculations in the SI section).

The concentration values on Table 2 are the average among the replicates. According to the reference values (ICP OES), the trueness was calculated and a range from 60 to 120% was considered as acceptable result. All the figures of merit mentioned are presented in Table 2, with normalization and signal type for each sample chosen according to acceptable results of trueness and RSD. The outstanding normalizations were selected according to the nearest trueness values of 100% and the range of slope was demonstrated in Table 2. The unknown concentrations for the samples were calculated using MEC (equation 1). For Ca, all the samples analyzed by LIBS showed values of trueness between 81 and 103% and values of RSD from 16 to 56%.

For sample S1, for example, the best results for Ca were obtained with normalization 9 (normalization by C I 193.09), but similar (trueness from 60 to 120%) results were also obtained for normalizations 1, 5, 10, 11 and 12. The obtained Ca concentration combining LIBS and MEC for sample S1 was 1.08% m m^{-1} with an uncertainty (95% of confidence level) of 0.18% m m^{-1}.

The IS method is applied to correct matrix-effect problems, but is currently used to improve the precision and accuracy if some variations during the analysis occur (i.e., transport, vapor generation, plasma temperature).[37,50] In addition, IS must have similar characteristics, such

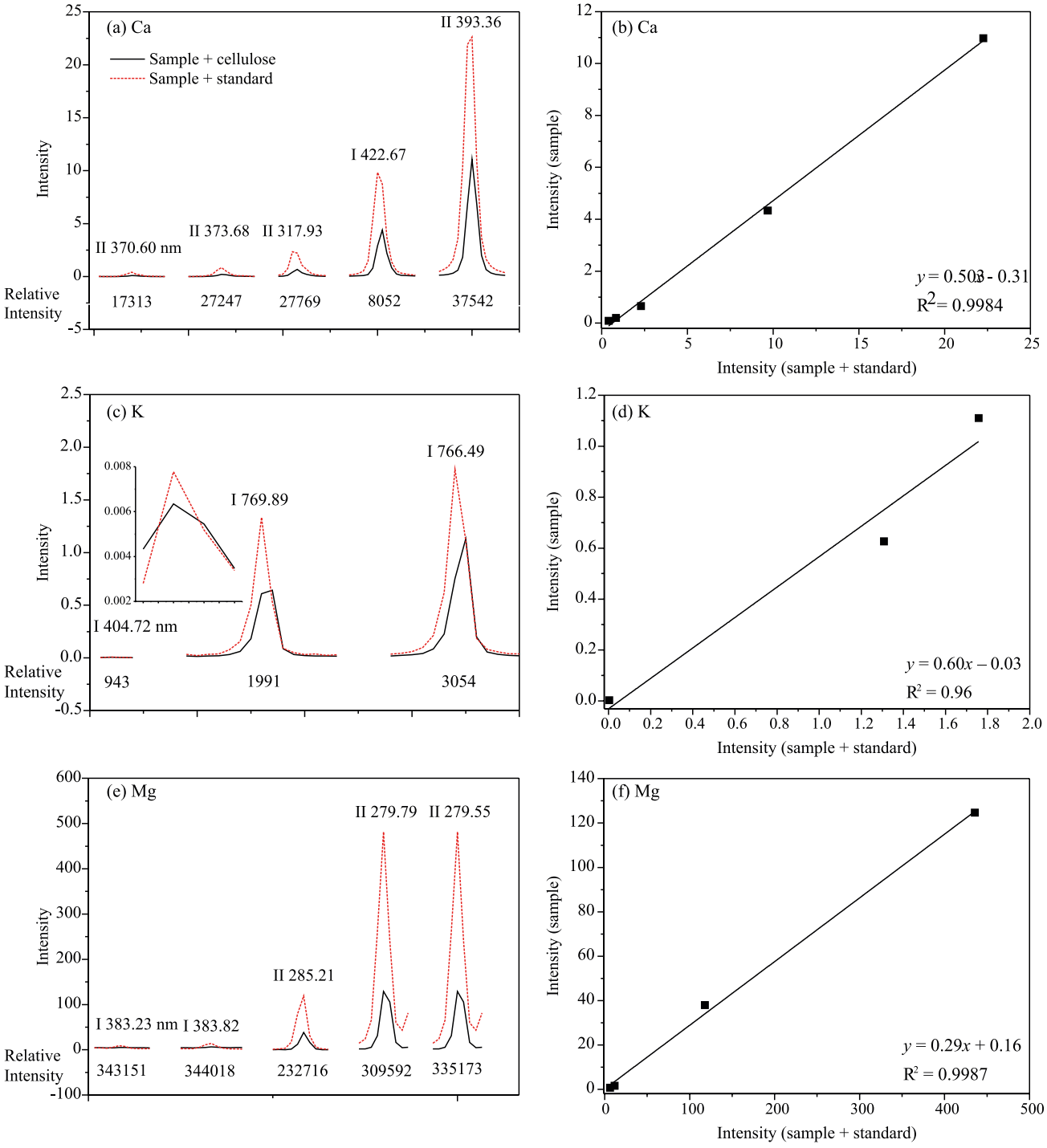

Figure 2. Selected and linear models for (a and b) Ca, (c and d) K and (e and f) Mg for sample 1.

as the atomic mass and/or first ionization potential and homogenous distribution in sample and standard material.[51] The selection and use of IS in solid analysis is not an easy task. In situations, as such the presented in this study, the use of elements naturally present in the sample composition is an alternative. All samples are carbon-rich due to the natural presence of this element and by addition of cellulose. In this sense, carbon was used as option for IS.[17] In Table 2, it is possible to note that the use of IS was satisfactory for Ca (normalizations 9, 10, 11 and 12). The other normalizations, including the signal average (1), presented unsatisfactory results with trueness values higher than 150% for the majority of the samples.

The same situation occurs for K, but only for the samples S3 and S5, with trueness of 95 and 109%, respectively. For the analyte K, there is a problem regarding

Table 2. Normalizations and figures of merit for the LIBS analysis using MEC as calibration method

Analyte	Sample	Data	Normalization type remark[a]	Slope range	LIBS[b] / (% m m^{-1})	Uncertainty	RSD / %	ICP OES / (% m m^{-1})	Trueness / %	Trueness range / %
Ca	S1	height	9: C I 193.09 nm (1, 5, 10, 11, 12)[c]	0.50-0.69	1.08	0.18	20	1.13	96	81-103
	S2	area	11: C I 247.85 nm (1, 4, 9, 10, 12)[c]	0.55-0.67	1.20	0.19	16	1.49	81	
	S3	height	9: C I 193.09 nm (1, 5, 10, 11, 12)[c]	0.39-0.43	0.49	0.23	47	0.49	100	
	S4	area	11: C I 247.85 nm (9, 10, 12)[c]	0.30-0.38	0.36	0.20	56	0.35	103	
	S5	height	11: C I 247.85 nm (2, 9, 10, 12)[c]	0.50 -0.59	0.90	0.21	23	1.02	88	
	S6	height	9: C I 193.09 nm (1, 5, 10, 11, 12)[c]	0.49-0.43	0.60	0.21	36	0.71	85	
K	S1	height	11: C I 247.85 nm	0.60-0.77	1.39	5.8	414	0.73	199	95-109
	S2	height	9: C I 193.09 nm	0.67-1.56	–2.19	0.97	45	1.23	–178	
	S3	area	5: sum (1)[c]	0.33-0.44	0.40	0.41	180	0.42	95	
	S4	height	11: C I 247.85 nm	1.10-1.71	–2.92	0.5	17	0.92	–317	
	S5	area	11: C I 247.85 nm (9, 10, 12)[c]	0.22-0.54	0.37	0.66	175	0.34	109	
	S6	area	11: C I 247.85 nm	0.71-0.89	2.71	0.56	20	1.43	189	
Mg	S1	height	8: individual spectrum maximum + sum (2, 4, 6)[c]	0.27-0.29	0.07	0.009	13	0.07	100	74-106
	S2	height	4: individual spectrum maximum + average (2, 3, 6, 7, 8)[c]	0.33-0.46	0.11	0.01	9	0.13	85	
	S3	height	2: norm and average (6)[c]	0.21-0.29	0.059	0.004	6	0.08	74	
	S4	height	10: C I 247.85 nm (2, 6, 9, 11, 12)[c]	0.36-0.40	0.11	0.02	16	0.13	85	
	S5	area	12: C I 247.85 nm (6, 9, 10, 11)[c]	0.45-0.49	0.16	0.03	22	0.16	100	
	S6	area	3: area and average (7)[c]	0.46-0.54	0.18	0.05	26	0.17	106	

[a]The numbers in parentheses refer to the normalizations 1 (average), 2 (norm and average), 3 (area and average), 4 (individual spectrum maximum and average), 5 (sum), 6 (norm and sum), 7 (area and sum), 8 (individual spectrum maximum and sum), 9 (C I 193.09 nm as internal standard (IS) and average), 10 (C I 193.09 nm as IS and sum), 11 (C I 247.85 nm as IS and average) and 12 (C I 247.85 nm as IS and sum). I and II refer to the atomic and ionic emission lines, respectively; [b]concentration calculated for LIBS using equation 1; [c]normalizations with trueness values in the range of 60 to 120%. LIBS: laser-induced breakdown spectroscopy; RSD: relative standard deviation; ICP OES: inductively coupled plasma optical emission spectrometry.

the emission lines, because only four are available in the studied spectral range (186-1042 nm). In this case, were possible to obtain only signals for the first two (Figure 2) most intense lines. The emission line 404.72 nm presented analytical signals with low signal-to-noise ratio (SNR). Even with the satisfactory results for the samples mentioned previously, no results for K are reliable, since the linear models has only 2 points.

From the eight emission lines tested for Mg, three presented spectral interferences (280.27, 517.27 and 518.36 nm) and were not considered for the linear model. For this analyte, the trueness values were from 74 to 106% and the RSD were from 6 to 26%. In this case, the best normalizations were not only for C as IS, but also for norms 2, 3, 4 and 8. All these values were extremely satisfactory, and matrix effect was not observed.

Differences between the atomization and ionization process of these three analytes (Ca, K and Mg) and C as IS, can be associated with the results.[52] As mentioned before, one condition to obtain an ideal IS are the similar values

of first ionization potential. For C the potential is 11.26 eV, greatly different from Ca, K and Mg (6.11-9.39 eV). This observation can be one of the reasons why C did not work so well as IS for Mg, but according to Kuznetsova and Morgulis,[53] just factors as the ionization potentials, excitation energies and similar thermal properties are not enough to have success using the IS.

As expected, all the slope values were below one and similar for Ca and Mg, ranging from 0.30 to 0.69 for Ca and from 0.24 to 0.48 for Mg. For K, the slope values were higher than those observed for Ca and Mg, with only two exceptions, S3 and S5 (samples that obtained acceptable trueness values). This observation can be due to the reduced number of emission lines observed for K.

A comparison between MEC and one-point gravimetric standard addition calibration (OP GSA) was performed. The OP GSA is a calibration method that approaches the same principle of MEC: matrix-matching, but in this case the method uses only one emission line, where the unknown concentration is calculated by curve extrapolation.[54,55]

The figure of merit values for the LIBS analysis using the OP GSA are shown in Table S3 (SI section). The emission lines that presented the best results in the MEC were also used for the OP GSA models; besides RSD and trueness, calculations of F test were performed to verify the curve linearity. Table S3 (SI section) shows the ratio of $F_{calculated}$ / $F_{tabulated}$. For Ca, the emission line 393.36 nm was better than the others, with trueness varying from 82 to 110% and RSD from 3 to 29%. For K (766.49 nm emission line), the trueness values were within the acceptable range only for S3 and S8 with 111 and 105% and RSD of 8 and 19%, respectively. The best emission line for Mg was 279.55 nm, where the trueness values varied from 82 to 108% and RSD from 4 to 8%, except for S2. The values of F-test ratio were high ($F_{calculated}$ higher than $F_{tabulated}$) showing that the OP GSA models are linear, except for Mg of S2 (ratio of 2) which presented a low trueness, being explained by lack of linearity.

The three analytes presented good results for the most intense emission lines: Ca 393.36, K 766.49 for few samples and Mg 279.55 nm for the majority of the samples. When these results are compared with MEC (see Table 2), the obtained one presented also good trueness for all samples and analytes. In addition, MEC showed a capacity to circumvent interferences related to matrix and spectral effects. This observation is clear for sample S2 when Mg was determined: the trueness for MEC and OP GSA were 85 (see Table 2) and 49% (see Table S3, SI section), respectively.

An additional test with different proportions of analytes into the stock mixture was proposed to evaluate if variations can negatively interfere and generate a matrix effect. Four stock mixtures were prepared and mixed with S1. A triplicate (n = 3) for each stock mixture with S1 was pelletized and analyzed by LIBS and ICP OES and the data was calculated following all the procedures proposed in this study. Table 3 shows the concentration values for each analyte and its ratio. The signal normalization modes selected were the same as described in Table 2.

Table 3. Normalizations and figures of merit for the LIBS analysis using MEC for sample S1 and different stock mixtures

Stock mixture	Concentration in stock mixture / (% m m^{-1})	Ratio among the analytes	Trueness / %
1	0.66 (Ca)	3.7	92
	0.68 (K)	3.7	–7793
	0.18 (Mg)	1	105
2	0.35 (Ca)	1.9	80
	0.60 (K)	3.3	120
	0.18 (Mg)	1	89
3	0.72 (Ca)	4	106
	0.26 (K)	1.4	–24
	0.18 (Mg)	1	104
4	0.72 (Ca)	8	92
	0.59 (K)	3.2	124
	0.09 (Mg)	1	81

The similarity of trueness values demonstrates that even changing the proportions of analytes in a stock mixture did not interfere in the results. In the specific case of Ca and Mg it was observed a good concordance with those results presented in Table 2. In the case of K, the results were not consistent due to the lack of emission lines available in the studied range.

Trueness values between 60 and 120% were obtained and, consequently, the efficiency of MEC with the spectral normalizations sort out matrix effect issues.

Conclusions

The use of MEC with LIBS is a suitable alternative of calibration for dietary supplements, due to matrix effect of these products. The results obtained for Ca and Mg were satisfactory with recovery between 60 and 120%. However, due to limitation of few emission lines for K, a reliable calibration model was not possible to obtain. For Ca, the possibility of using C was successfully exploited improving the precision and accuracy, and for Mg other normalizations also improved these figures of merit. In general, with MEC and the normalization, it is possible to observe spectral interferences in the linear models, avoid

matrix effect due to matrix matching and improve results of precision and trueness.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) PhD grants 2014/11415-0 to A. S. A. and 2016/17221-8 to J. P. C., and grant 2016/01513-0. The authors are also grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) grants 401074/2014-5, 305637/2015-0 and PhD grant 160152/2015-1 to M. A. S. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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Submitted: June 22, 2018
Published online: October 22, 2018

Supplementary Information

Combination of Multi-Energy Calibration (MEC) and Laser-Induced Breakdown Spectroscopy (LIBS) for Dietary Supplements Analysis and Determination of Ca, Mg and K

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Table S1. Microwave heating program applied for sample mineralization

Step	Power / W	Temperature / ºC	Ramp time / min	Holding time / min
1	1260	120	5	5
2	1260	160	5	5
3	1260	230	5	10

Table S2. ICP OES instrumental conditions to obtain reference values for Ca, K and Mg

Parameter	Operational condition
Integration time for low emission line / s	15
Integration time for high emission line / s	5
Sample introduction flow rate / (mL min^{-1})	4.2
Sample flow rate during the analyses / (mL min^{-1})	2.1
Pump stabilization time / s	5
Radio frequency applied power / W	1150
Auxiliary gas flow rate / (L min^{-1})	0.5
Nebulization gas flow rate / (L min^{-1})	0.5
Cooling gas flow rate / (L min^{-1})	12
Lines for Ca, K and Mg on axial and radial view / nm	Ca (II 393.366), K (I 769.896) and Mg (II 280.270)

I and II: atomic and ionic emission lines, respectively.

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Table S3. Figures of merit for the LIBS analysis using one-point gravimetric standard addition calibration (OP GSA)

	Ca				K				Mg			
Sample	λ / nm	RSD / %	Trueness / %	Ratio[a]	λ / nm	RSD / %	Trueness / %	Ratio[a]	λ / nm	RSD / %	Trueness / %	Ratio[a]
S1	λ1(393.36)	29	94	16	λ1(766.49)	46	196	–	λ2 (383.829)	9	45	–
S2		13	87	24		–141	–211	–		51	28	–
S3		3	110	64		8	111	44		82	24	–
S4		4	99	41		–43	–320	–		6	46	–
S5		15	83	87		19	105	6		14	52	–
S6		3	82	83		36	194	–		12	26	–
S1	λ3 (317.93)	29	34	–	λ2(769.896)	31	116	–	λ3 (383.23)	14	35	–
S2		17	42	–		–161	–170	–		35	23	–
S3		7	39	–		12	97	–		21	32	–
S4		14	35	–		–46	–322	–		16	40	–
S5		17	36	–		21	92	–		7	56	–
S6		3	32	–		39	206	–		22	23	–
S1	λ4 (373.687)	20	27	–	–	–	–	–	λ4 (279.55)	4	107	8919
S2		14	49	–		–	–	–		415	49	2
S3		7	46	–		–	–	–		6	87	539
S4		5	83	–		–	–	–		6	82	462
S5		20	45	–		–	–	–		6	98	612
S6		3	31	–		–	–	–		8	108	340
S1	λ6 (370.603)	21	26	–	–	–	–	–	λ6 (279.79)	4	107	–
S2		14	47	–		–	–	–		322	52	–
S3		11	50	–		–	–	–		6	87	–
S4		30	49	–		–	–	–		6	82	–
S5		10	43	–		–	–	–		6	97	–
S6		6	34	–		–	–	–		11	107	–
S1	λ7 (422.673)	23	71	–	–	–	–	–	λ8 (285.21)	4	124	–
S2		14	98	–		–	–	–		–195	–1	–
S3		4	134	–		–	–	–		7	78	–
S4		4	135	–		–	–	–		7	62	–
S5		9	68	–		–	–	–		7	62	–
S6		4	95	–		–	–	–		9	66	–

[a]Ratio of $F_{calculated}$ / $F_{tabulated}$. RSD: relative standard deviation.

Calculation of the uncertainty

In this part of the Supplementary Information, we will use an example to explain how to calculate the uncertainty slope of the linear model and how to propagate this value to the final results.

The first step is the organization of the matrices. In this example, we will use replicate number 2 for sample S1 and for Ca determination. As five emission lines were used (see Figure 2a for more details), an **X** matrix containing five rows and two columns is need. The first column contains the Ca signal intensity for the five emission lines for pellet 2 (sample + stock mixture, see Figure 1), and is used to calculate the slope (b_1), and the second column "ones" to calculate the intercept (b_0). The coefficients (b_1 and b_0) are calculated according to:

$$b = (X^t X)^{-1} \times X^t y \qquad \text{(S1)}$$

where y is the Ca signal intensity for pellet 1 (sample + cellulose, see Figure 1).
The error for each coefficient is calculated according to:

$$\text{error} = \sqrt{(MS_{\text{residue}}(X^t X)^{-1})} \qquad \text{(S2)}$$

where $MS_{residue}$ is the mean of squares of the residues.
The uncertainty for the coefficients will be:

$$\text{uncertainties} = \text{error} \times t \qquad \text{(S3)}$$

where t value was selected with 95% of confidence.
In the specific case of the above-mentioned sample, the slope (b_1) value was 0.503 and the uncertainty was 0.037.
The relative uncertainty was calculated according to:

$$\text{Relative uncertainty} = \frac{0.0369}{0.5028} = 0.0735 \qquad \text{(S4)}$$

These calculations should be applied to all the replicates and the propagated relative uncertainty was calculated using the equation S5.

$$\text{relative } \sigma = \sqrt{\text{relative } \sigma 1^2 + \text{relative } \sigma 2^2 + \text{relative } \sigma 3^2} \qquad \text{(S5)}$$

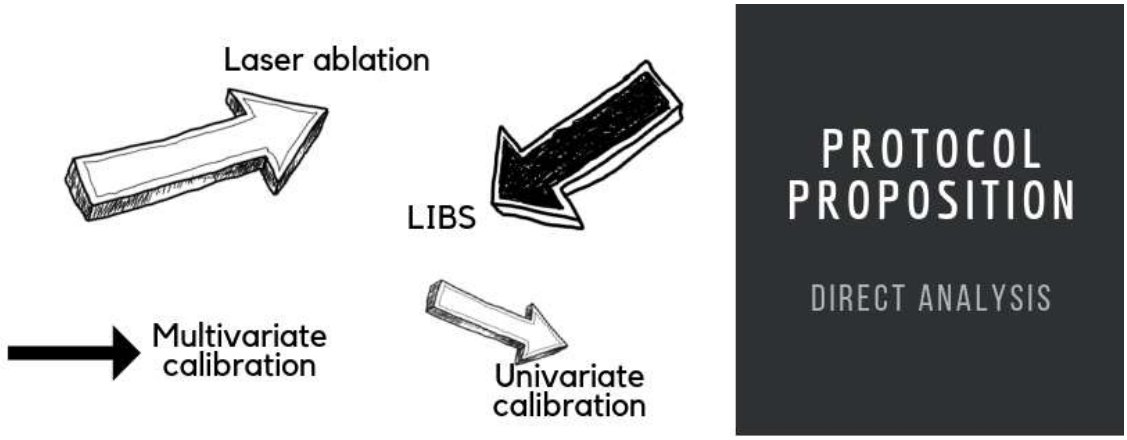
where relative σ is the propagated relative uncertainty.
These calculations were repeated for all samples and the final results are presented in Table 2.

Chapter 5 – Unpublished Results

5. Unpublished Results

5.1"Protocol proposition for the direct analysis of solid samples by Laser Ablation-Inductively Coupled Optical Emission Spectroscopy (LA-ICP OES) and Laser-Induced Breakdown Spectroscopy (LIBS): Powdered dietary supplements example"

Submitted



Protocol proposition for the direct analysis of solid samples by Laser Ablation-Inductively Coupled Optical Emission Spectroscopy (LA-ICP-OES) and Laser-Induced Breakdown Spectroscopy (LIBS): an example using powdered dietary supplements

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Abstract

This study suggests an analytical protocol for direct solid analysis by laser-ablation based techniques of macro and micronutrients in food samples, in particular, powdered dietary supplements. Two laser-based techniques were used: Laser-Induced Breakdown Spectroscopy (LIBS) and Laser-Ablation Inductively Coupled Plasma Optical Emission Spectroscopy (LA-ICP-OES) in tandem mode. A total of 6 samples of powdered dietary supplements were used as test samples. Sixty-six additional samples were prepared by mixing these samples with cellulose to produce standards for the calibration models. Two quantification approaches were explored: one based on classical linear models and a second one based on a multivariate calibration model.

Keywords: Tandem (LA/LIBS), protocol, direct solid analysis, dietary supplements, elemental analysis

Introduction

Routine analysis of solid samples by conventional analytical techniques such as Flame Atomic Absorption Spectroscopy (FAAS), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) typically requires the conversion of the solid samples to a homogeneous liquid solution for analysis. However, there are several limitations to solid samples dissolution approaches, as (1) several procedures are time-consuming, in some cases from hours to days; (2) use of toxic chemicals, for example, organic solvents (xylene, benzene, etc.), or strong acids alone or in combination (aqua regia, etc.), as well as in some cases the use of hydrogen peroxide. Additionally, in every step of solid samples digestion, there is an increased chance of contamination or loss of an element of interest. Moreover, significant concerns arise due to the generation of chemical waste.[1]

Since the introduction of laser-based techniques, such as Laser-Induced Breakdown Spectroscopy (LIBS) in the 1960s, and Laser Ablation ICP-MS (LA-ICP-MS) / Laser Ablation ICP-OES (LA-ICP-OES) in the 1980s, the possibility of direct solid analysis for fast multi-elemental qualitative and quantitative analysis started to be exploited by the analytical chemistry community.[2,3]

LIBS is an attractive approach due to its instrumental simplicity, in-situ analysis capabilities, high sample throughput, and access to every element in the periodic table, while Laser Ablation for ICP-MS is attractive due to its sensitivity and access to isotopic information.[2,4-8] These approaches to direct chemical analysis of solid samples are straightforward as both start when a high-power pulsed laser is focused on the sample surface for material removal and subsequent plasma formation. From this laser-induced plasma, atomic, ionic and molecular emission lines can be measured which is the basis of LIBS.[4,5] Then as the plasma

cools down, solid particles start to form, and these particles could be directed into a second ionization source such as an ICP-(OES or MS) for analysis.[3,9,10] As can be deducted from this chain of events (starting with the laser-material interaction), the collection of signals from these techniques occur sequentially therefore, independent measurements can be made from the same laser-ablation event without interfering with each other. There are other significant motivations on why to use these techniques in tandem, for example, information obtained are complementary, especially with regards to expanding concentration coverage, from weight percentage to parts per million (%wt and mg kg^{-1}) usually reported for LIBS, to sub-mg kg^{-1} for LA-ICP based measurements. Additionally, elemental coverage increased by providing the capability of detection of all the elements in the periodic table.[11–13] Therefore, a laser ablation based tandem system provides enhanced capabilities to measure all elements and with greater dynamic range.

Notwithstanding the significant advantages for the direct analysis of solid samples, the direct quantitative chemical analysis of solids still facing some challenges. In particular, the need for matrix-matched standards. The matrix-matched standard requirement exerts an immediate impact on the quantification strategies suitable for the analysis, requiring the use of standards reference materials with chemical and physical matrix close, in composition and behavior, to the unknown samples. These matrix effects become a significant issue when standards reference materials are not commercially available for a particular set of questioned samples. In those cases, in which reference materials cannot be readily found, some labs had reported the successfully use of in-house fabricated standards.[14-16]

This study provides a closer look at the steps of protocol development for direct analysis of solid samples by laser-based techniques, from selection (and preparation) of standards for calibration, questioned sample preparation, and data

analysis strategies. To achieve these goals the present study is focused on the development of a quantitative analysis step-wise protocol for direct solid analysis of powdered dietary supplements using a LIBS/LA-ICP-OES combined technique.

Experimental

Samples

We selected six powdered dietary supplement samples for this protocol development. These samples were purchased from local markets as they were intended to be consumed by children and young adults. A significantly increased interest in these products has been reported during the last few years. For example, in the United States, about 50% of adults and 30% of children are using or had used dietary supplements.[17-19] Based on this information, an increased concern about the quality of these products with regards to elemental contaminants, levels of metals and vitamins content has emerged. Therefore, quality control of these products is necessary. We labeled the six samples in this study as: S1, S2, S3, S4, S5, and S6, for anonymity.

Instruments and Methods

The first step of this study was to mineralize these six samples and analyzed them by a conventional technique, in this case, we performed liquid analysis by ICP-OES (iCAP 6000, Thermo Scientific, Waltham, MA, USA) in order to obtain reference values. The samples were mineralized by acid digestion, employing a closed vessel microwave system (Speedwave Four, Berghof, Eningen, Germany). The procedure encompasses the use of 250 mg of sample treated with 6 mL of HNO_3 (2 mol L^{-1}). Initially, the concentrated HNO_3 was purified using a sub-

boiling distillation system DistillacidTM BSB-939-IR (Berghof, Eningen, Germany). After digestion, the final volume was adjusted to 14 mL with deionized water. All the reagents used were of analytical grade or higher purity. The deionized water used to prepare the solutions was 18.2 ΩM cm resistivity and produced by a Milli-Q® Plus Total Water System (Millipore Corp., Bedford, MA, USA).

Table 1 shows the experimental conditions for the microwave heating program used for sample preparation and ICP-OES measurements instrument conditions. The standards used for the calibration of these analyzes were multi-element standard solutions prepared daily from 10000 mg L^{-1} Ca along with 1000 mg L^{-1} K and Mg stock solutions (Qhemis, Jundiaí, SP, Brazil).

Table 1: Microwave heating program and ICP-OES parameters

Speedwave Four, Berghof (total of 3 steps)		
Power (W)		1260/1260/1260
Temperature (°C)		120/160/230
Ramp time (min)		5/5/5
Hold time (min)		5/5/10
ICP-OES iCAP 6000 Thermo Fisher Scientific		
Characteristics		Parameters
Integration time for low and high emission lines (s)		5
Sample introduction flow rate (mL min^{-1})		2.1
Sample flow rate during analyses (mL min^{-1})		2.1
Pump stabilization time (s)		25
Radio frequency applied power (W)		1200
Auxiliary gas flow rate (L min^{-1})		0.25
Nebulization gas flow rate (L min^{-1})		0.83
Cooling gas flow rate (L min^{-1})		16
Emission lines (nm) for the analytes	Axial	Ca II 317.933
		Mg II 279.553
	Radial	Cu I 324.754
		Fe II 238.203
		K I 769.896
		Zn II 202.548

For direct solid sample analysis, we used a J200 Tandem (LA/LIBS) system from Applied Spectra, Inc. This system is equipped with a Nd:YAG nanosecond pulsed laser at 213 nm wavelength. The ablation chamber could accommodate samples up to 100mm diameter with flexibility in volume and washout time. This system was interfaced with the 5100 SVDV-ICP-OES (Agilent, Santa Clara, CA, USA). Table 2 shows a list of the optimized conditions used in these experiments. Laser ablation was performed in He as the carrier gas and Ar as a make-up gas before entering the ICP-OES plasma. The samples were ablated using a laser repetition rate of 10Hz while moving the sample at speed 0.1 mm s^{-1}. Using the Agilent ICP-OES software ICP-Expert, signals were acquired in the time-resolved analysis mode (TRA). The transient signals from LA-ICP-OES were integrated using the Clarity software by Applied Spectra, Inc.

Table 2: Experimental condition for tandem LIBS-LA-ICP-OES measurements

ICP-OES Agilent 5100	
Power (W)	1200
Plasma Ar gas flow rate (L min^{-1})	12
Auxiliary Ar gas flow rate (L min^{-1})	1
Make-up He gas flow rate (L min^{-1})	0.70
Viewing mode	SVDV
Working wavelengths (nm)	C I 193.027, Ca I 422.673, Cu I 324.754, Fe II 234.350, K I 766.491, Mg II 280.270, Zn II 202.548
Laser Ablation System J-200 Applied Spectra	
Laser wavelength (nm)	213
Laser pulse energy (mJ)	4
Repetition rate (Hz)	10
Pre-ablation time (s)	15
Laser Delay (s)	25
Scan speed (mm s^{-1})	0.1
Carrier He gas flow rate (L min^{-1})	0.5 L/min
Spot size (μm)	125
Number of lines	10
Acquisition mode	Accumulated

Results and Discussion

Six elements were the focus of the liquid samples analysis by ICP-OES: Ca, Cu, Fe, K, Mg, Zn. The calculated reference concentrations (n=3) from these analyses will be used as target values for the direct solid analysis, Table 3. Each analysis protocol consisted of three fundamental steps, specifically: sample preparation, data collection, and data analysis. Each one of these steps presents decision making challenges mainly due the large number of options available. We describe in detail options available and some of the challenges encounter during analysis protocol. The final goal was to turn the decision-making process simpler. For this purpose, we established decision trees for some of the junctions encountered along the way of the protocol development process.

Table 3 Reference values by ICP-OES (mg kg^{-1})

	S1	S2	S3	S4	S5	S6
Ca 317.933nm	12849±221	14309±583	4431±340	2924±96	11457±716	12232±59
K 769.896 nm	7518±285	10573±425	2512±287	5715±108	2478±87	3745±165
Mg279.553nm	805±20	1432±66	779±64	1275±69	2043±174	2551±26
Fe 238.203nm	178±38	246±39	232±13	279±20	162±9	169±24
Zn 202.548nm	70±1	81±5	182±14	184±16	107±12	114±3
Cu 324.754nm	9.8±0.3	12.9±0.5	5.6±0.2	9.3±0.5	1.5±0.7	2.7±0.7

Figure 1, shows a general decision tree as an overview of the entire process of protocol development. Figure 2, shows a more detailed example of the sample preparation step. For this study, the original form of the commercial samples was coarse powder, and as indicated in the decision tree in Figure 2, we decided to pelletize these samples. One of the reasons for this decision was that in comparison to direct powder ablation, a compact pellet will provide more reproducible ablation sampling, as it has been well documented.[20,21]

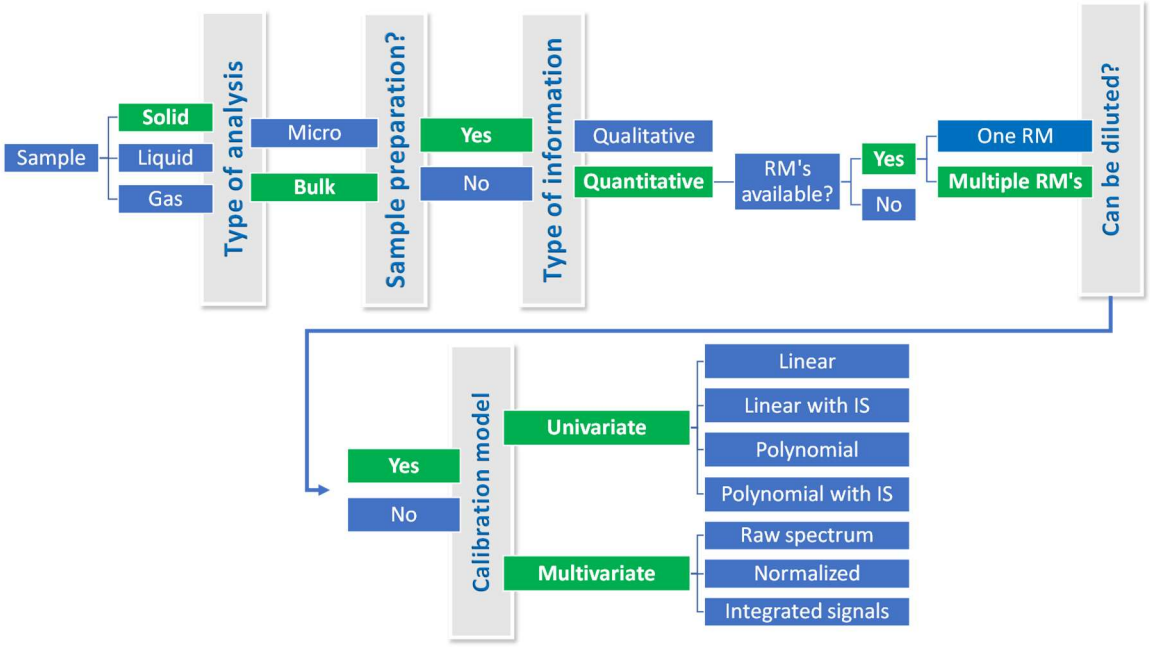


Figure 1: General decision tree

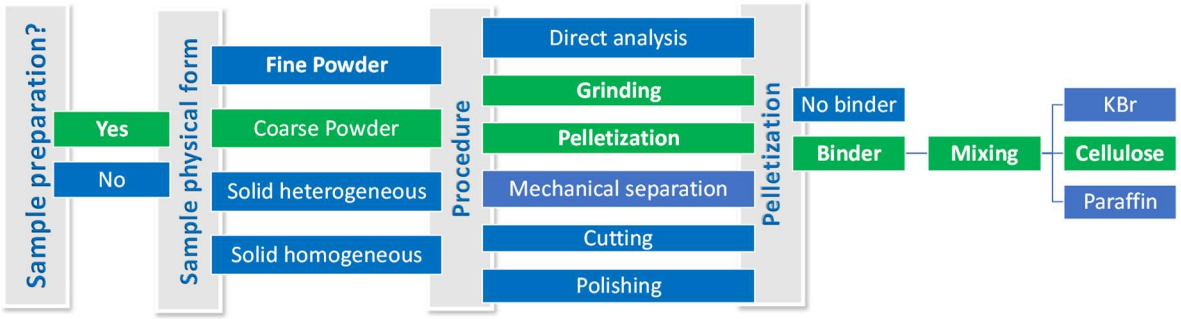


Figure 2: Sample preparation decision tree

However, direct palletization of these type of coarse samples will produce heterogenous pellets, to test this assumption, a portion of the samples was ground to fine powders using a mixer mill (Spex, model 8000D). The grounding of the original samples resulted in a reduction and homogenization the particle size. The resultant finer powders were pelletized, as well as six of the original samples without grinding. Figure 3, shows images acquired by a handheld microscope 5 MP- Dino-Lite Edge of the pellets before and after mechanical homogenization.

The pellets were analyzed by LA-ICP-OES using the optimized conditions (Table 2). The results are presented in Tables 4 and 5. Table 4, shows the comparison between the correlation coefficient values (R^2), for each element, from linear regression (univariate calibration) for the two sets of samples.

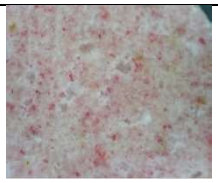
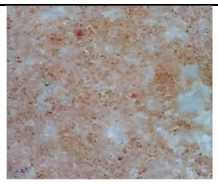
Before	Samples identification	After
	S1	
	S2	
	S3	
	S4	
	S5	
	S6	

Figure 3: Samples pellets without and with mechanical homogenization

It is clear that the correlation values (R^2) display improvements for four of the six analytes, except K and Fe. In the case of K, the difference is not as evident when compared to the difference displayed by Fe. Iron behavior could be attributed to the weak signal obtained from both sets of samples due to the low concentration

obtained (see Table 3). Additionally, relative standard deviations (RSD) values from the homogenized samples were systematically lower when compared to those that were not homogenized (Table 4).

Table 4: Correlation (R^2) and RSD range values to the pure samples without and with homogenization

Analyte and emission line	Without Homogenization		With Homogenization	
	R^2	RSD (%) range	R^2	RSD (%) range
Ca 422.673 nm	0.91	5 - 20	0.99	2 – 7
Mg 280.270 nm	0.86	7 – 32	0.96	2 – 11
K 766.491 nm	0.97	5 – 26	0.90	3 – 6
Cu 324.754 nm	0.62	20 – 129	0.83	11 – 17
Fe 234.350 nm	0.15	8 - 30	-0.48	8 – 36
Zn 202.548 nm	0.62	15 – 92	0.97	6 – 26

Based on these results it was evident that a better approach should involve each sample to be ground into a fine powder before palletization. We also decided to mix these new fine powders with cellulose. Cellulose serves multiple purposes: (1) acting as a binder to produce stronger pellets and help improve the relative standard deviation, (2) as a solvent to dilute the original sample (solute) which allows us to prepare solid samples of different concentrations, and (3) the carbon from the cellulose could be used as an internal standard (IS). Therefore, after grinding the original samples, they were mixed with cellulose at different proportions, from 10 to 90%wt with increments of 10%wt, where 0%wt refers to only cellulose and 100%wt refers to pure sample after homogenization of the particles. These combinations were made using a mixing mill for 5 min to ensure

homogenization and then pelletized using 7 tons of pressure with an automatic pressing machine model 3630 from Spex. From this process, we produced a total of 66 pellets split as follows: 6 pure samples, 54 mixed samples and 6 cellulose blanks. Precaution was taken from using multiple pelletization containers to assure cleanliness after every use.

The second step of protocol development was data collection. This step involved selection of the analytical technique and optimization of the experimental parameters. Usually, an analytical method is selected on the basis of sample form, elements to be determined and their expected concentrations. In this study, we were interested in evaluating laser ablation-based techniques for the direct analysis of these solid samples. Therefore, the techniques options were confined to LIBS, LA-ICP-OES or LA-ICP-MS. The J200 tandem LIBS/LA system is capable of performing these techniques simultaneously (LIBS + LA-ICP-MS or LIBS + LA-ICP-OES). The main advantage of this approach is that a tremendous amount of time can be save from the overall process by reducing a two-step process (potentially two separate analysis) to a single one in which two independent techniques analysis is being done simultaneously.

There were several reasons for the selection of LA-ICP-OES as the primary technique in this study; specifically a) ICP-OES was used to analyze the liquid version of the same samples, and b) ICP-OES is regularly used for food and environmental samples inspection in liquid form or after sample digestion, as there are several regulatory for water analysis i.e. EPA (200.5, 200.7, and 6010), ISO (11885:007), etc. Additionally, in this study the simultaneous measurement by LIBS will allow us to evaluate this other option as a possibility for future analysis of some of the elements of interest with high sample throughput. Once the technique(s) (LA-ICP-OES, LIBS) were selected, the experimental conditions were optimized, in this case, laser pulse energy, spot size, repetition rate, ablation

mode, gas environment, etc. based on signal-to-noise ratio of the elements of interest. Table 2, shows the optimized experimental conditions for direct solid analysis by LA-ICP-OES and LIBS simultaneously.

The third step of the protocol development is data analysis. The selection is usually dictated by the analysis performed (qualitative or quantitative), the type of information that is pursued, and the ablation mode, i.e. bulk analysis for average composition of components of the sample, or microanalysis in any of its forms: depth profiling, inclusion analysis, chemical mapping. Finally, data form, data set size, etc. will dictate how the data are ultimately reduced and the useful information extracted. For this study, we want to determine the bulk concentration of a few elements from these samples. Both techniques provide raw optical emission spectra, or after processing individual elements their signal intensities can be extracted. If data analysis starts directly from the raw spectra, data reduction could be less time consuming and could also yield to reduced bias data due to limited influence of analyst input. However, this approach could add unintended noise to the analysis ultimately deteriorating precision. Alternatively, if we start from the integrated emission lines, we could be more selective with the information included in the calibration models. We could, for example, minimize noise and deal with interferences. However, this option requires a closer involvement of the analyst in the selection of the lines, interference correction approaches, etc. The general decision tree in Figure 1 shows some of the options available and explore in this study.

Calibration models:

Performing external calibration using matrix-matched standards is the most common approach in laser ablation-based analysis,[3] and in general, the use of

certified reference material is preferred.[13,22-24] However, the use of matrix-matched standards prepared in-house is a common practice in the laser ablation community.[14,16] These in-house standards are usually made by mixing solid compounds, i.e., as salts or by diluting a pure sample using a binder then mixing and pressing (as is the case in this study). Also, by preparing samples from Borate fusion, in some cases, the addition of standard solutions to the powered matrix followed by drying, pressing or immobilization process has been reported.[14,16, 25-28] For the dietary supplements, there were not (to the best of our knowledge) certified reference materials available for external calibration. Therefore, we used in-house standards prepared from diluting the original samples with a binder (cellulose).

In order to stablish a calibration model 32 samples were selected, and six as "test samples" for protocol validation. For the "calibration set" we wanted to cover the broader possible range of concentrations, but we also wanted to keep the number of samples included in the calibration model to a realistic quantity 4 or 5 samples per set. The criteria for the "test samples" were to have them be close to the middle of the calibration curves concentration range. Each set of calibration samples contained the following number of samples: S1 four samples, S2 four samples, S3 five samples, S4 four samples, S5 five samples, and S6 four samples, plus a blank for each set (pure cellulose). Table 5 shows the standards selected for the direct solid analysis and the respective concentration values.

Univariate models for each "calibration set" were built based on the sampling grouping mentioned above, on the other hand for the multivariate calibration model all 32 samples in the calibration set were used, producing a multivariate calibration model with contribution of all samples simultaneously. As shown in the decision tree, Figure 1, there are two main data analysis methods based on univariate and multivariate analysis.

Table 5: Calibration standards: identification and concentration (mg kg^{-1})

		Ca	Mg	K	Cu	Fe	Zn
S1 (4 srm's)	Blank	0	0	0	0	0	0
	S1_20%	2649	166	1550	2.0	37	14
	S1_70%	8838	554	5171	6.8	122	48
	*S1_80%	10126	634	5925	7.7	140	55
	S1_90%	11476	719	6715	8.8	159	62
S2 (3 srm's)	Blank	0	0	0	0	0	0
	S2_30%	4274	428	3158	3.8	73	24
	S2_50%	7067	707	5222	6.4	122	40
	*S2_60%	8533	854	6305	7.7	147	48
	S2_100%	14309	1432	10573	12.9	246	81
S3 (4 srm's)	Blank	0	0	0	0	0	0
	S3_20%	934	164	530	1.2	49	38
	*S3_40%	1785	314	1012	2.3	93	74
	S3_60%	2657	467	1507	3.4	139	109
	S3_90%	3985	701	2259	5.1	209	164
	S3_100%	4431	779	2512	5.6	232	182
S4 (3 srm's)	Blank	0	0	0	0	0	0
	S4_60%	1758	766	3435	5.6	168	111
	S4_70%	2034	887	3976	6.5	194	128
	*S4_90%	2619	1142	5118	8.4	250	165
	S4_100%	2924	1275	5715	9.3	279	184
S5 (4 srm's)	Blank	0	0	0	0	0	0
	S5_10%	1174	209	254	0.2	17	11
	S5_20%	2383	425	515	0.3	34	22
	*S5_30%	3548	633	767	0.5	50	33
	S5_50%	5662	1010	1225	0.7	80	53
	S5_80%	9071	1617	1962	1.2	128	85
S6 (3 srm's)	Blank	0	0	0	0	0	0
	S6_10%	1243	259	381	0.3	17	12
	*S6_30%	3717	775	1138	0.8	51	35
	S6_40%	4936	1029	1511	1.1	68	46
	S6_100%	12232	2551	3745	2.7	169	114

*Test sample used for validation

Classical univariate methods are usually based on calibration curves built using single emission line intensity *versus* the concentration. Then the elemental content of the "test samples" is calculated from the fitting equation to either linear or polynomial regression models. This approach is susceptible to matrix effects due to the fact that a single variable is being use to describe the behavior of an analyte. A conventional way to compensate matrix effects is normalizing to an internal standard, in this particular case we decided to use carbon as an internal standard, which has been reported to have mixed results.[7,13,16,26,29-31] In this case, we justify this selection for two main reasons: (1) dietary supplements contain a high concentration of C, and (2) cellulose (used here as a binder) main component is carbon ($C_6H_{10}O_5$).

The second approach consists of multivariate analysis of the data. This type of analysis involves observation and analysis of more than one variable at a time. These multivariate methods are useful when several factors contribute to the overall observed response. Some of the most mainstream approaches are Partial Least Square Regression (PLS-R),[32,33] Multi-Linear Regression (MLR),[33] and Principal Component Regression (PCR).[34]

In this study, the calibration models tested were the following: Univariate linear model, Univariate linear model with an internal standard (IS), and Multivariate model from signal intensities (ICP-OES) and raw spectra (LIBS) with normalizations.

We used PLS-R analysis for LIBS and LA-ICP-OES data. The raw spectra from LIBS were normalized using 12 different normalizations schemes.[35] These normalizations schemes are applied with the objective to minimize sample microheterogeneity and shot-to-shot signal fluctuation during data acquisition. After the normalization was applied, PLS-R calculations were performed for each

set of normalized spectra. The whole LIBS spectral profile which contains 12288 variables was mean-centered. Then the Regression Vectors calculated were inspected, and the most relevant emission lines for each analyte were selected. Matlab (MathWorks, Natick, Massachusetts, USA) version 2017b, was used for the normalization of the spectra and the Pirouette Multivariate Data Analysis software, version 4.5 (Infometrix, Bothell, WA, USA), was used to select the emission lines and calculate the PLS-R calibration models. For all the PLS-R calibration models a cross-validation leaving-on-out was applied, the latent variables (LV), standard error of validation (SEV) and prediction values were obtained.

Eighteen, six and nine emission lines were selected for Ca (315.887, 317.993, 393.366, 396.847, 422.673, 585.745, 612.216, 616.216, 643.907, 644.981, 646.256, 647.166, 649.379, 714.851, 720.219, 732.615, 820.172 and 824.880 nm), K (404.414, 404.721, 691.108, 693.878, 766.490 and 769.896 nm) and Mg (277.983, 279.078, 279.553, 279.799, 280.27, 285.213, 516.732, 517.268 and 518.361 nm), respectively. However, due to the low concentration in these samples of Cu, Fe and Zn it was difficult, and in some cases impossible, to detect their emission lines with the used configuration in this study. After completing the step of lines selection, PLS-R models were again calculated for Ca, K and Mg with the mean-centered emission lines intensity selected.

Similar approach was use for the LA-ICP-OES data, however because in this case we use all the integrated signals for PLS-R the normalization schemes were not applied. A PLS-R was calculated for each analyte using only its respective selected lines (lines with visible intensity signals). Multivariate calibration model (PLS-R with the 32 samples) were built for Ca, Cu, Fe, K, Mg an Zn without IS, and using the integrated signal of C 193 nm and C 247 nm as IS.

A few metrics were used to describe these experimental results. For the univariate and linear approach, we used the percentage of recovery (trueness) as a measure of the accuracy, relative standard deviation (RSD) as a measurement of the precision, and the correlation coefficient (R^2) of the linear fitting as a measure of the calibration model quality. The desire situation is a recovery between 80 and 120%, a low RSD value (typically lower than 10%) and a R^2 value near 1.

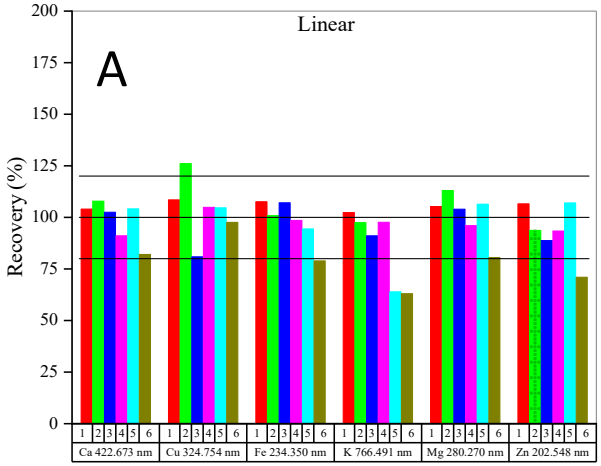
For the multivariate approach, we used the percentage of recovery as a measure of the accuracy, latent variables (LV), standard error of validation (SEV) as a measure of the calibration model quality. In the next two sections we present the results obtained with LA-ICP-OES and LIBS, respectively.

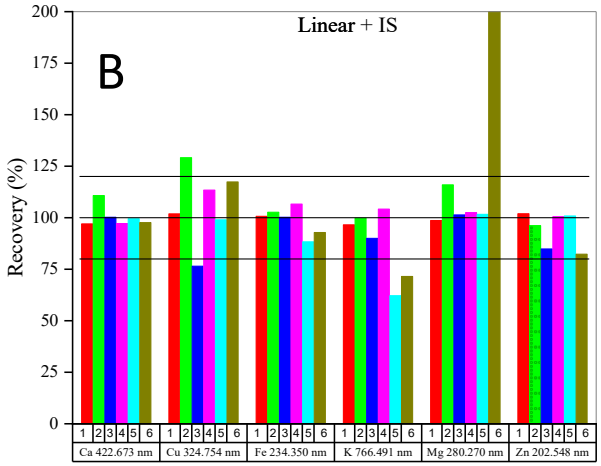
LA-ICP-OES

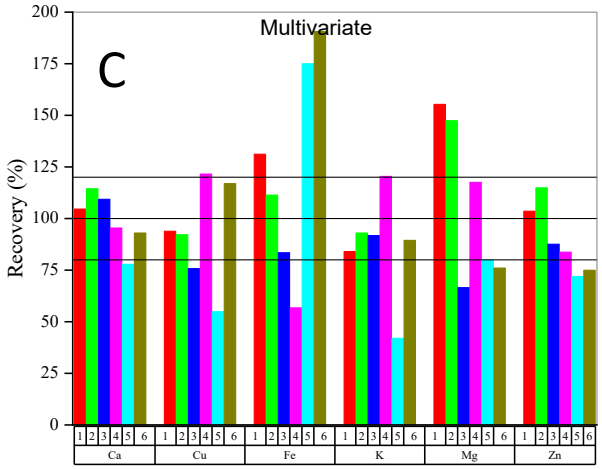
The integrated signals of Ca, Mg, K, Cu, Fe, and Zn from LA-ICP-OES were used to build the calibration models. Figure 4 shows the results of the six sets of samples in terms of the figures-of-merit from these calibration models. Row one in Figure 4 shows the results of the recovery percentage for the univariate methods (A and B) and for the multivariate approach (C). Row two shows the results of the relative standard deviation in percentage (D and E) and row three shows the correlation coefficient (R^2) (F and G) for the linear models.

The linear models (including the one that uses C as internal standard) displayed mixed results, except for Ca and Mg, where the six test samples were within the +/- 100% recovery range. When using an internal standard, the sample from batch of S6 (S6-30%) is out of the defined threshold. This sample (exception for Ca, Cu and Mg) consistently produces results outside the range while samples set numbers two and five produce mixed results. The two sets of samples that produced the best results are sets number one and four. These two sets are composed of samples whose concentration is evenly distributed compared to the

other calibration sets. Similarly, for set number six, which exhibits the worse performance, the test sample (S6-30%) is not close to the mean of the calibration curve.


Linear
A
Recovery (%)
200
175
150
125
100
75
50
25
0
1 2 3 4 5 6
Ca 422.673 nm
Cu 324.754 nm
Fe 234.350 nm
K 766.491 nm
Mg 280.270 nm
Zn 202.548 nm


Linear + IS
B
Recovery (%)
200
175
150
125
100
75
50
25
0
1 2 3 4 5 6
Ca 422.673 nm
Cu 324.754 nm
Fe 234.350 nm
K 766.491 nm
Mg 280.270 nm
Zn 202.548 nm


Multivariate
C
Recovery (%)
200
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150
125
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Ca
Cu
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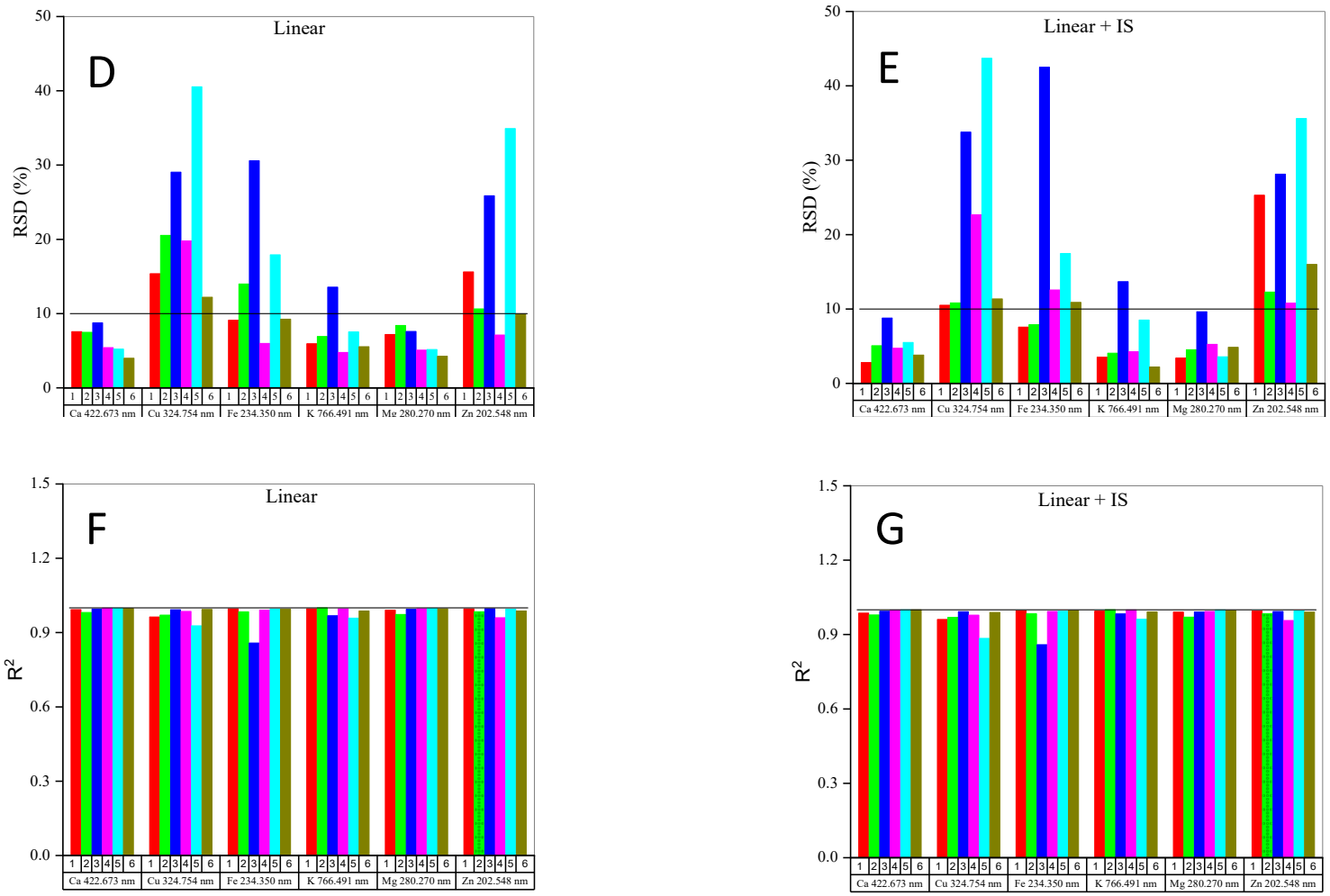


Figure 4: Comparison between calibration approaches and figures of merit from LA-ICP-OES results

Therefore, there is correlation between the position of the test samples with respect to the standards in the calibration curve. These results are not surprising because is well known that the most reliable results are obtained close to the centroid of the calibration curve.

Even with the use of C as an IS, no definitive improvement was obtained for this approach. Based on these measurements C does not qualify as a good candidate for internal standards for these samples in the case of univariate calibration. The fact that these food products contain complex structures (lipids, proteins, carbohydrates, etc.) which are additional sources of carbon signal, reduces the efficacy of its use as an internal standard. These complex structures also increase matrix effects which typically affect individual emission lines compared to the spectra as a whole.

Other noticeable trends from the linear model's results are the higher relative standard deviation for the micro elements (Cu, Fe and Zn), this fact may be due the low concentration values more affected when the samples are not totally homogenized. For the Ca, K and Mg all the RSD values were below 10%, with an exception for the sample 3 for K.

For the LA-ICP-OES data, the recovery values of these univariate calibration models were satisfactory as desired (within $\pm$20% of the know value) for almost all samples. They varied from sample set-to-sample-set in some cases, and from element-to-element mainly depending on the samples selected as standards. Therefore, careful selection of the standards combination and conditions for each analyte must be exercise in order for this approach to work correctly.

In this study we used the values predicted after cross validation in order to calculate square error of cross validation (SECV). This value gives a good estimate on how the model performs for unknown cases. The SECV was obtained by a process where the number of latent variables (LV) was changed. A plot of SECV

versus LV was used to determine the optimized number of latent variables. These latent variables have the best predictive power for each element. For the multivariate models on the LA-ICP-OES data the number of latent variables for each element were: 1 for Ca, Mg, Fe and Zn, and 2 for K and 3 for Cu. The values of SECV for each calibration model (one for each analyte with the 32 samples in each) were: 1096, 1767, 301, 1.51, 73 and 17 mg kg^{-1} for Ca (integrated signal with C 247 nm as IS), K (integrated signal with C 247 nm as IS), Mg (integrated signal with C 247 nm as IS), Cu (integrated signal with without IS), Fe (integrated signal without IS) and Zn (integrated signal with C 247 nm as IS), respectively.

The multivariate model also displayed mixed results for all the analytes when the validation set was used (Figure 4), to be considered a satisfactory multivariate model the values of SEV (standard error of validation) needs to be lower of the concentration values of the samples, preferably this difference should be of 3-fold lower.

For K, for example, the sample concentrations values for the S5-30% and S6-30% were below the SEV which makes the prediction unreliable. Concentrations values lower or closer to the SEV for Mg (all samples), Cu (S3-40%, S5-30% and S6-30%), Fe (all samples, exception for S4-90%) and Zn (S2-60%, S5-30% and S6-30%) also make these results unreliable.

Calibration for LIBS data

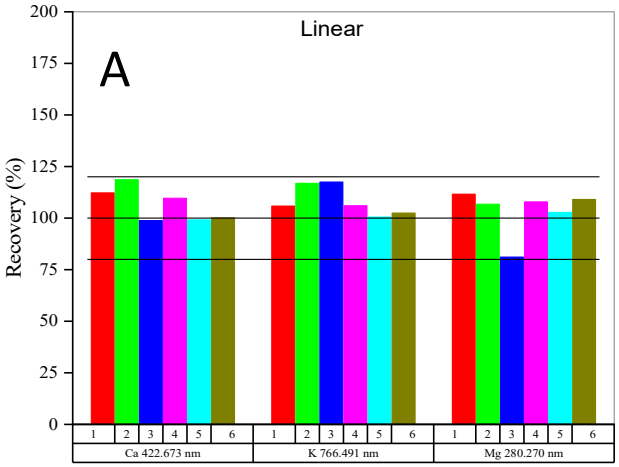
The proposed calibration models also were applied to the LIBS data, Figure 5. However, in this case, the univariate linear model (with and without internal standard) were only built for Ca, K, and Mg due to the low signal values for Fe, Cu, and Zn from some of these samples.

LIBS data display mixed results for all the samples, but even that, for the linear models without IS, the recovery values were between 81 to 119%, and

precision averaged below 10 %RSD for most samples. These results did not show improvement when the linear model with internal standard was applied. For the macro elements the linear approach provided satisfactory (recovery values into the range of 80 to 120%) results for all tested samples when compared with the LA-ICP-OES.

As mentioned before for the LIBS data the raw spectra were normalized, after the normalizations and selection emission lines, from PLS-R, the multivariate models were proposed for each analyte (PLS-R). The values of SECV were evaluated in order to select the lowest values and consequently the best normalizations for each model.

For Ca the best normalization was when the C 193 nm was used as IS, in this case was considered 5 LV and the SECV was 878 mg kg^{-1}. Only 2 LV were used in the model for K and the SECV value was 569 mg kg^{-1}, for this analyte the normalization applied was the calculation of individual the norm and average.[35] For Mg, 3 LV were selected and the SEV value was 297 mg kg^{-1}, the best normalization was the individual spectrum maximum and average.[35]


Linear
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Recovery (%)
200
175
150
125
100
75
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25
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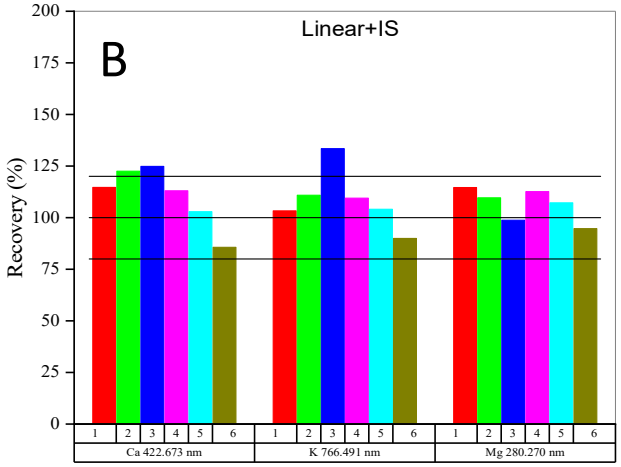
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Ca 422.673 nm
K 766.491 nm
Mg 280.270 nm


Linear+IS
B
Recovery (%)
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1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Ca 422.673 nm
K 766.491 nm
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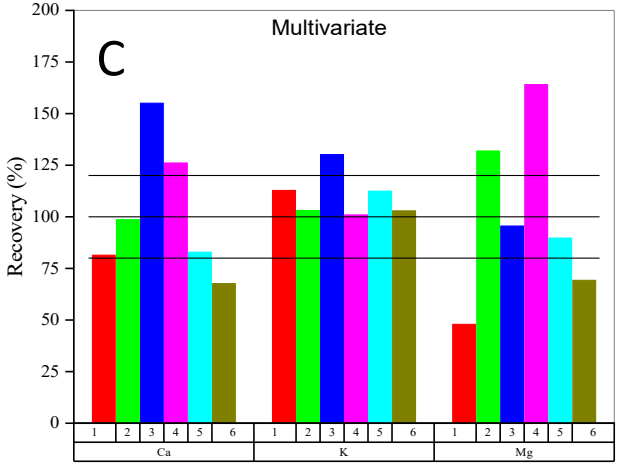
Multivariate
C
Recovery (%)
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Mg

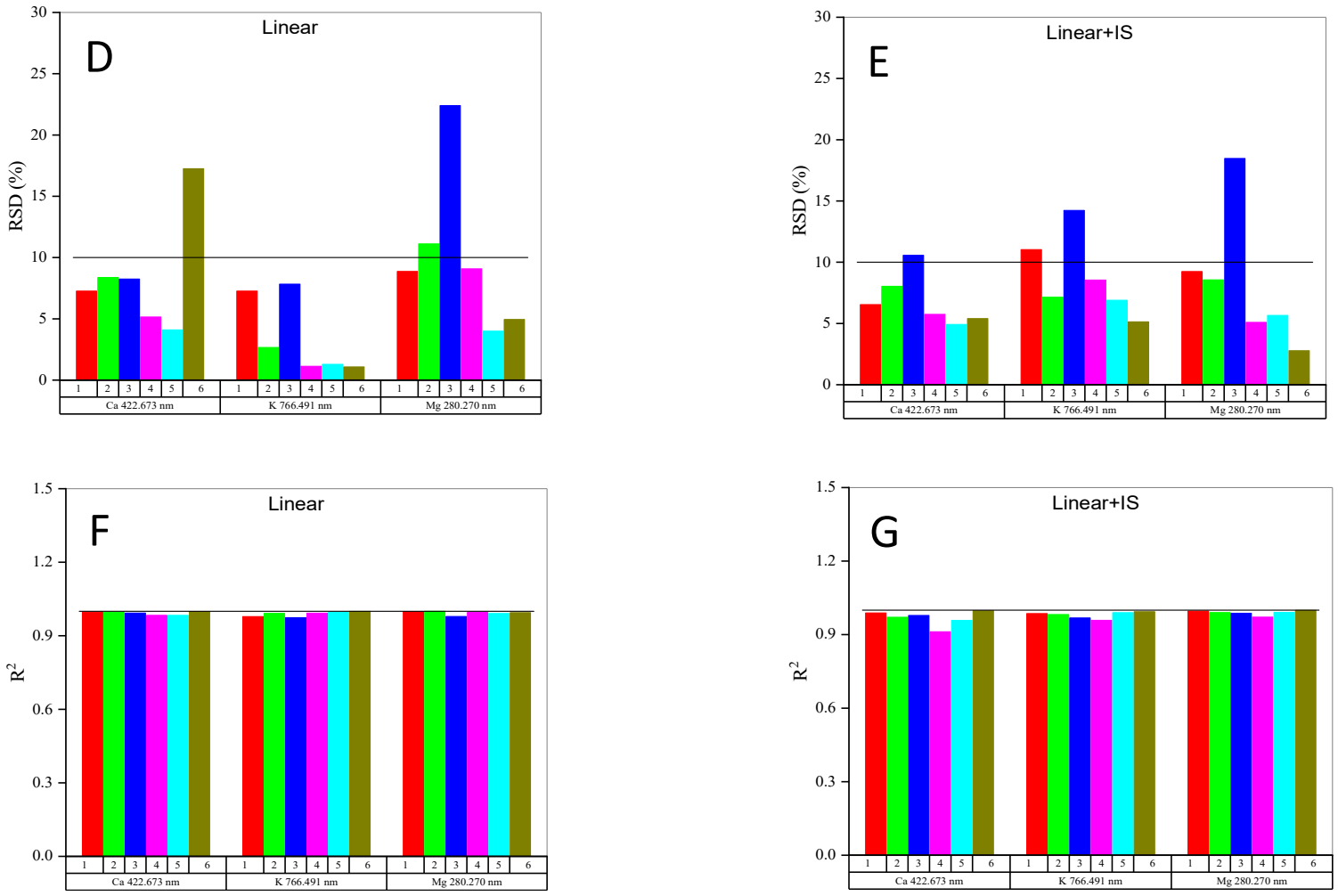

Figure 5: Comparison between calibration approaches and figures of merit for LIBS results

Even with all the normalizations process the results of the multivariate models for LIBS data was similar to the LA-ICP-OES, in this case the higher values of SECV also affected the prediction of the "test samples". LIBS results were similar in performance compared to the results obtained by LA-ICP-OES. However, the results from LA-ICPOES were a little higher (for Ca, K and Mg) in terms of the recovery values from 63% to 108% (linear model). For LIBS data the samples number two and three consistently were positioned outside the threshold of acceptable results for all the elements.

Conclusions

In this study, we delivered a step-by-step view of protocol development for direct solid analysis by laser-based techniques of powdered dietary supplement samples. We confirmed an approach to sampling preparation to facilitate the production of improved and robust samples for direct solid analysis by laser-ablation methods, as well as demonstrated that reliable standard materials can be prepared and used for quantification of these dietary supplements samples. We also showed a comparison between two laser-based techniques (LA-ICP-OES and LIBS with measurements taken simultaneously) demonstrating that both laser-based techniques are suitable for this type of analysis, which saves a significant amount of time and effort, in particular in the initial stages of the protocol development, which is also particularly useful when attempting to find which technique best suit the analysis needs. And finally, a comparison between three calibration models was made. The comparison revealed that some of the matrix effects are too strong to be compensated by the internal standard selected in this study (carbon), both of the calibration model (linear and PLS-R) showed mixed results which means that more optimization of the protocol for analysis of these samples is necessary. Next

step in the protocol development will involve a more exhaustive selection of the samples for the calibration set as well as determination of the best (minimum) number of emission lines selected for construction of the calibration models. We also plan to refine the steps presented here and apply to different types of samples.

Acknowledgment

This research has been supported by the Office of Nuclear Nonproliferation, U.S. Department of Energy under Contract No. DE-AC02-05CH11231 and by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), project number 2016/17443-0. The authors are also grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – grant 305637/2015-0)

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Chapter 6 – Conclusions

6. Conclusions

In this thesis, it was possible to demonstrate several approaches for the quantitative analysis of food samples by LIBS, LA-ICP OES and LA-ICP-MS. The use of laser techniques for quantitative and direct solid analysis was proved as a suitable alternative. Different calibration strategies and laser techniques (LIBS, LA-ICP OES and LA-ICP-MS) were applied for analysis of dietary supplements, powder milk and orange juice, and the potential of these techniques and drawbacks were extensively explored as proposed initially. Also, other remarks may be highlighted:

- The external calibration with in-house standards and multivariate calibration strategy showed good alternatives for the dietary supplements and powder milk analysis by LIBS;
- The external calibration with in-house standards for the dietary supplements and orange juice was applied in LA-ICP-MS analysis. Several spectral and matrix interference problems were faced and compromised significantly the trueness values;
- The low RSD values, below of 20%, for orange juice samples (LA-ICP-MS) showed the successful homogenization process with PVA for the liquid analysis, however it was not possible the concentrations quantification for Fe, Cu, Cd and Pb (concentration values below the LOQ);
- The mixture of PVA and orange juice also were analyzed by LIBS, but it was not possible to detect intensity signals for any analytes;
- The DLLME strategy was applied to improve the LOQ for the LIBS analysis, but although it was possible optimize an ideal condition for the DLLME it did not present satisfactory results (minimum or nonexistent intensity signal) when applied to the juice samples analysis by LIBS;
- New calibration strategies as MEC and OP GSA improved the results for the dietary supplements and can be explored as a good option for future studies with direct analysis. The MEC and OP GSA showed similar values of trueness for

all the analytes and samples, but some exceptions proved the efficiency of the MEC compared to the OP GSA;

- A protocol of analysis of direct solid samples by a tandem LA/LIBS system was proposed and a step-by-step evaluated, dietary supplements samples were used as example and the protocol was successful applied;
- Due the complexity of the food samples and the difficult of direct solid analysis some process of normalization showed an important contribution to achieve best results in all the results demonstrated (published and unpublished results);
- The LIBS, LA-ICP OES and LA-ICP-MS drawbacks were explored as well as different calibration strategies;
- For the macro elements analysis, the LIBS technique and the MEC strategy demonstrated promising results for quantitative and direct food analysis.